ELECTRONICALLY FILED ON APRIL 25, 2008 **PATENT**

Previous Docket No. 07149-1307
Attorney Docket No. 36671-716.505

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

In re Application Inventor: Kenneth F. Buechler Application No.: 09/613,650 Filed: July 11, 2000 Title: DIAGNOSTIC DEVICES AND APPARATUS FOR THE CONTROLLED MOVEMENT OF REAGENTS WITHOUT MEMBRANES	Confirmation No.: 9972 Art Unit: 1797 Examiner: Lyle Alexander Customer No. 021971

RESPONSE TO NOTIFICATION OF NON-COMPLIANT APPEAL BRIEF

MAIL STOP APPEAL BRIEF - PATENTS
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

This Response to Notification of Non-Compliant Appeal Brief is in response to the Notification of Non-Compliant Appeal Brief (37 CFR 41.37) dated March 25, 2008. An Evidence Appendix and a Related Proceedings Appendix accompany this Response.

Remarks begin on page 2 of this paper.

REMARKS

The Notification of Non-Compliant Appeal Brief states that the Brief filed March 10, 2008 does not contain copies of the evidence submitted under 37 C.F.R. §§ 1.130, 1.131, or 1.132 or of any other evidence entered by the Examiner and relied upon by Appellant in the appeal, along with a statement setting forth where in the record that evidence was entered by the Examiner, as an appendix thereto. In addition, the Notification indicates that the Brief does not contain copies of the decisions rendered by a court or the Board in the proceeding identified in the Related Appeals and Interferences section of the Brief as an appendix thereto.

Appellant provides herewith in Appendix B, an Evidence Appendix, and in Appendix C, a Related Proceedings Appendix. Thus, Appellant believes that the Brief has been appropriately corrected.

CONCLUSION

All of the objections in the Notification have been addressed and the Brief is believed compliant.

Please grant any necessary Petition for Extension of Time necessary for filing this Response. The Commissioner is authorized to charge any additional fees that may be required, including Petition fees and Extension of Time fees, to Deposit Account No. 23-2415 (Docket No. 36671-716.505).

Respectfully submitted,

WILSON SONSINI GOODRICH & ROSATI

Date: April 25, 2008

By: _____
Chantal Morgan D'Apuzzo, Ph.D.
Reg. No. 48,825

650 Page Mill Road
Palo Alto, CA 94304-1050
(650) 320-4840
Customer No. 021971

Appendix B: Evidence Appendix

1. U.S. Patent 4,647,543 (Stöcker) was cited by the Examiner in the non-final Office Action mailed on April 14, 2005.

2. U.S. Patent 5,091,318 (Anawis) was cited by the Examiner in the non-final Office Action mailed on July 13, 2007.

3. U.S. Patent 5,445,970 (Rohr) was cited by the Examiner in the non-final Office Action mailed on July 13, 2007.

4. U.S. Patent 5,458,852 (Buechler) was cited by the Examiner in the non-final Office Action mailed on July 13, 2007.

5. U.S. Patent 6,019,944 (Buechler) was cited by the Examiner in the non-final Office Action mailed on July 13, 2007.

United States Patent [19]

Stöcker

[11] **Patent Number: 4,647,543**

[45] **Date of Patent: Mar. 3, 1987**

[54] **PROCESS FOR ANALYSES TO BE CARRIED OUT ON IMMOBILIZED BIOLOGICAL TISSUE**

[76] Inventor: **Winfried Stöcker**, Krummesserweg 3, D-2419 Rondeshagen, Fed. Rep. of Germany

[21] Appl. No.: **582,394**

[22] Filed: **Feb. 24, 1984**

[30] **Foreign Application Priority Data**

Feb. 25, 1983 [EP] European Pat. Off. 83101863

[51] **Int. Cl.**[4] **G01N 1/28**; G02B 21/34

[52] **U.S. Cl.** **436/174**; 424/3; 435/284

[58] **Field of Search** 424/3; 250/461.2; 350/536; 356/246; 436/172, 174, 503; 435/284, 808, 240

[56] **References Cited**

U.S. PATENT DOCUMENTS

2,965,219	12/1960	Rhodin	350/536
3,556,633	1/1971	Mutschmann	350/536
3,720,470	3/1973	Berkhan	350/536
4,003,707	1/1977	Lübbers et al.	436/172
4,339,241	7/1982	Stöcker	422/68
4,353,856	10/1982	Mück et al.	424/3
4,387,164	6/1983	Hevey et al.	436/166 X
4,404,181	9/1983	Mauthner	424/3
4,435,508	3/1984	Gabridge	435/284

FOREIGN PATENT DOCUMENTS

1133598	3/1961	Fed. Rep. of Germany	424/3

OTHER PUBLICATIONS

Ames Division, Miles Laboratories; N-Multistix Reagent Strips (product publication) 1981.

Kawahara et al; Bonding Compositions to the Hard Tissue of Human Body; Chem. Abst. CA 94:197574, 1981.

Reusable Glass-Bound pH Indicators, Harper, Anal. Chem. V 47, No. 2, pp. 348-351.

Histopathologic Technique and Practical Histochemistry Lillie, 1965, p. 98.

Chemical Abstract (CA) 67(13): 1795h, Boyer, 1967.

CA 98(19): 157312Z, Huang et al, 1983.

CA 98(18): 148461V, Svoboda et al, 1982.

Aloe Scientific Catalog No. 103, 1952, p. 650.

The Section Freeze-Substitution Technique: I. Method, Chang et al, J. Histochem. & Cytochem. vol. 9, 1961.

Primary Examiner—Barry S. Richman

Assistant Examiner—Michael S. Gzybowski

Attorney, Agent, or Firm—Nixon & Vanderhye

[57] **ABSTRACT**

Processes for testing immobilized biological material with generally biochemical and histochemical methods, particularly enzyme, immuno and hormone chemical methods can be improved by adhering the biological material to the surface of a support and then fixing the support to a plate. The plate is constructed in such a way that the biological material is protected from damage. Using a biological testing technique such as thin section immunofluorescence testing the invention makes it possible to carry out biochemical tests in a more rational and trouble-free manner than with hitherto known methods. If necessary, a random number of tests can be performed side-by-side on a single plate. The invention makes it possible to simply and rapidly prepare frozen section products and store them in a space-saving manner at very low temperatures. The adhesion of the frozen sections to the support can be improved by coupling chemicals which react with the tissue by bonding thereto to the support.

13 Claims, 33 Drawing Figures

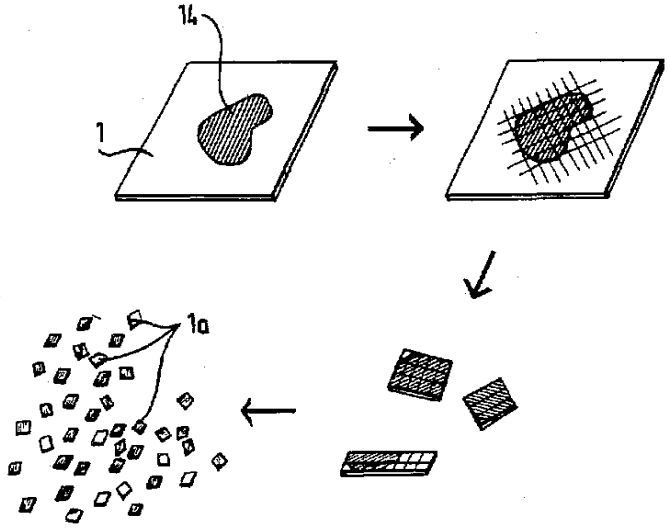


FIG. 1A

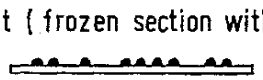


FIG. 1B

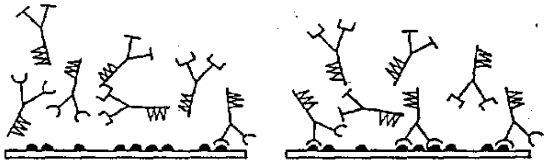


FIG. 1C

FIG. 1D

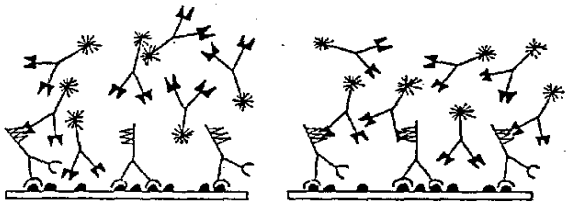


FIG. 1E

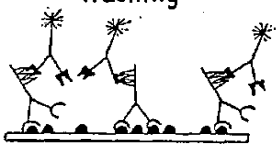


FIG. 2

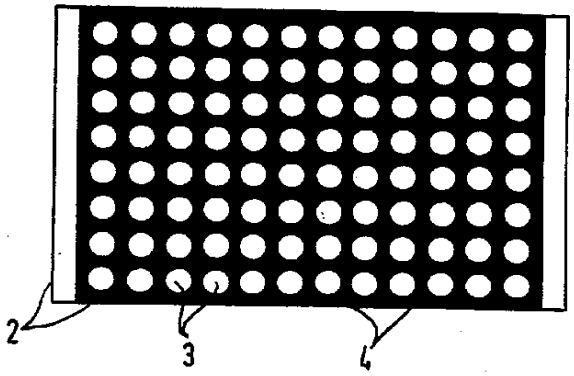


FIG. 3

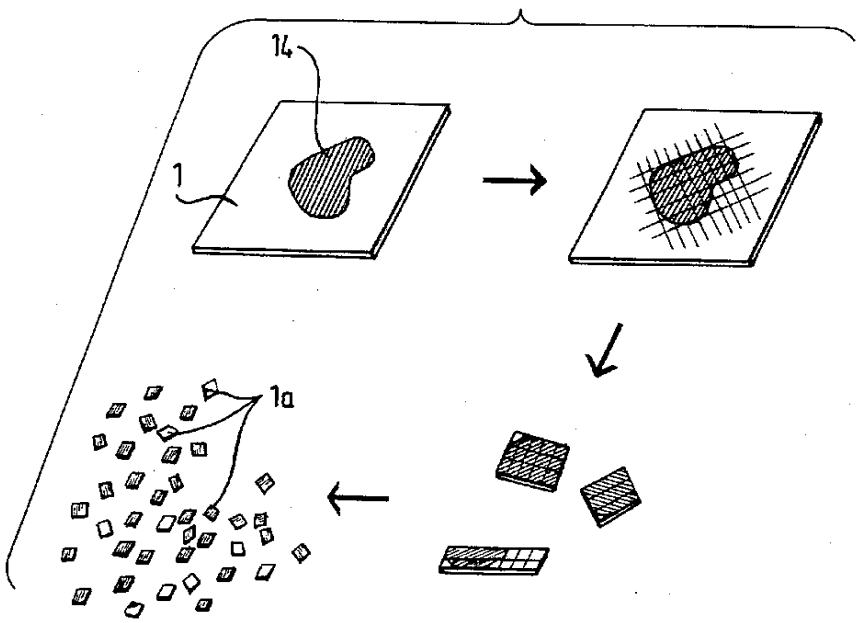


FIG. 4a

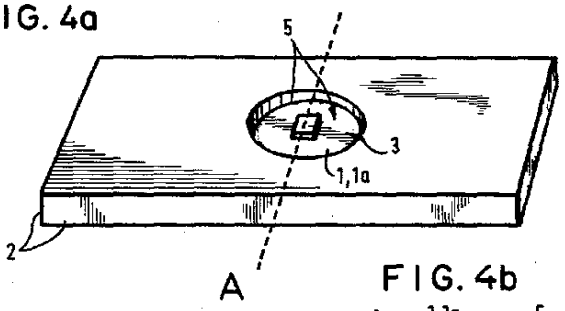


FIG. 4b

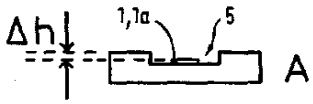


FIG. 4c

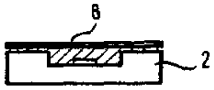


FIG. 6a

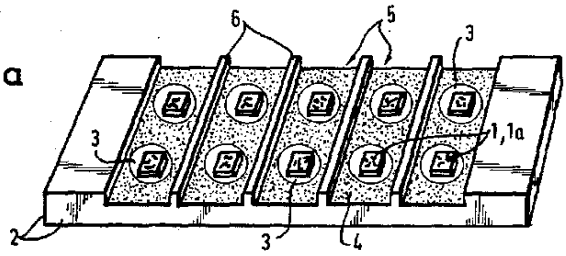


FIG. 6b

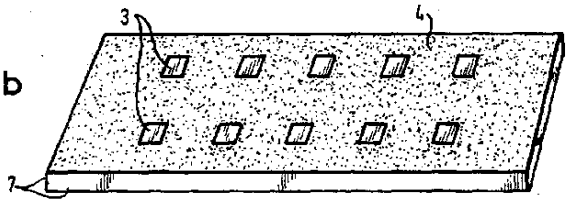


FIG. 5

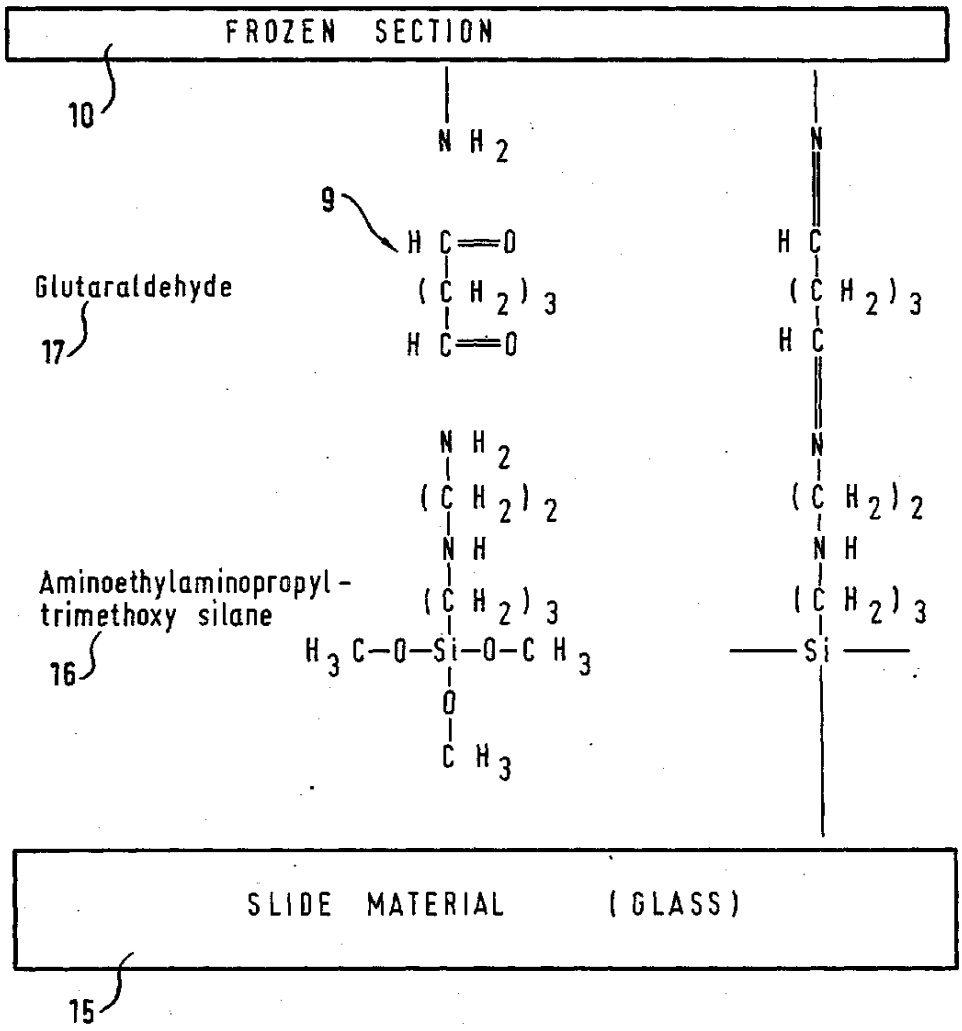

FROZEN SECTION
10
9
Glutaraldehyde
17
Aminoethylaminopropyl-trimethoxy silane
16
SLIDE MATERIAL (GLASS)
15

FIG. 7

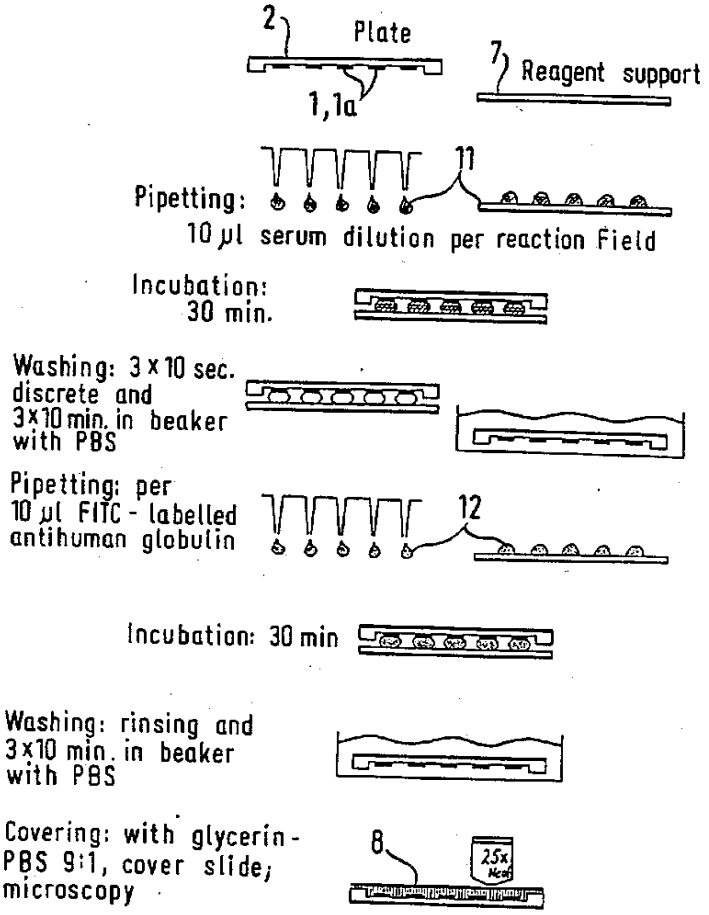

2
Plate
7
Reagent support
1,1a
Pipetting:
11
10 µl serum dilution per reaction field
Incubation:
30 min.
Washing: 3 x 10 sec.
discrete and
3x10 min. in beaker
with PBS
Pipetting: per
10 µl FITC - labelled
antihuman globulin
12
Incubation: 30 min
Washing: rinsing and
3x10 min. in beaker
with PBS
Covering: with glycerin -
PBS 9:1, cover slide;
microscopy
8
25x

FIG. 8a

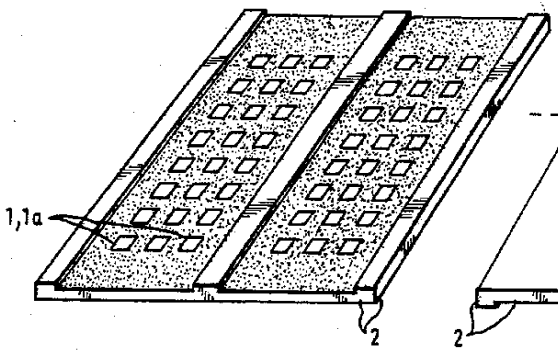


FIG. 8b

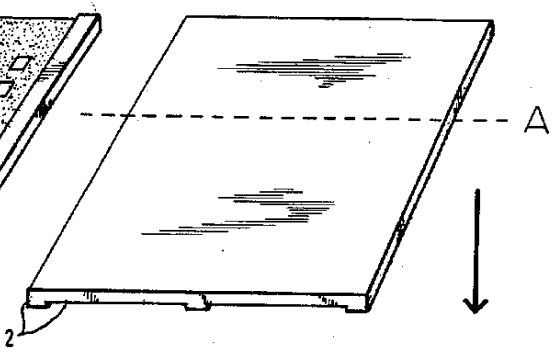
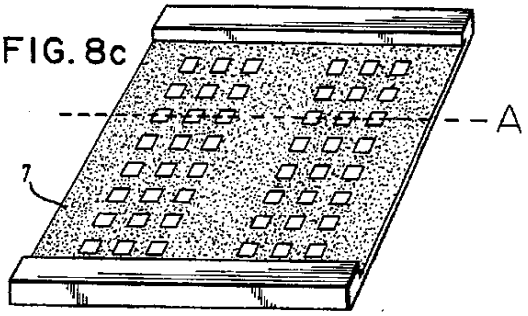


FIG. 8d

FIG. 8c



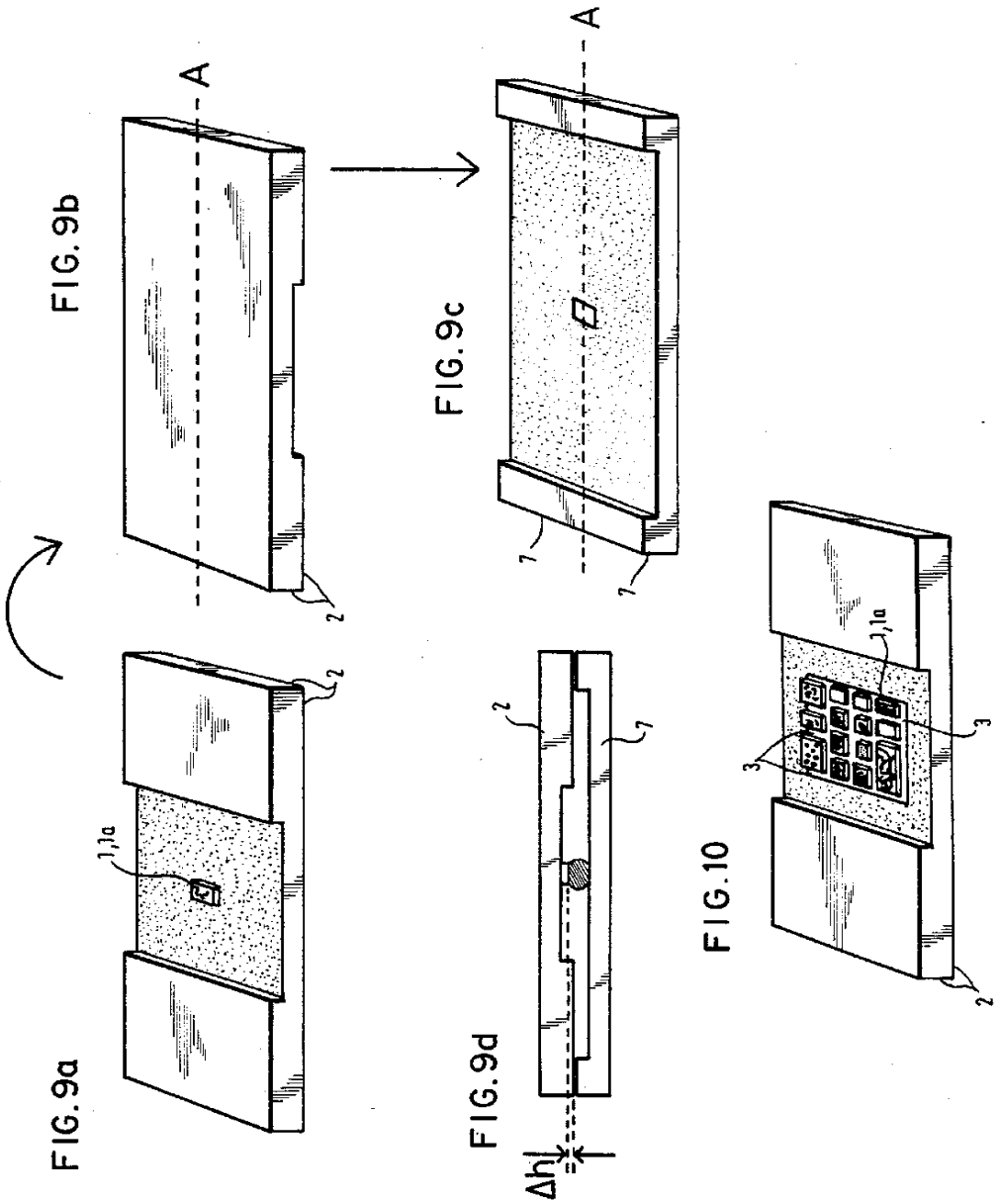


FIG. 9b

FIG. 9c

FIG. 9a

FIG. 9d

FIG. 10

FIG. IIa

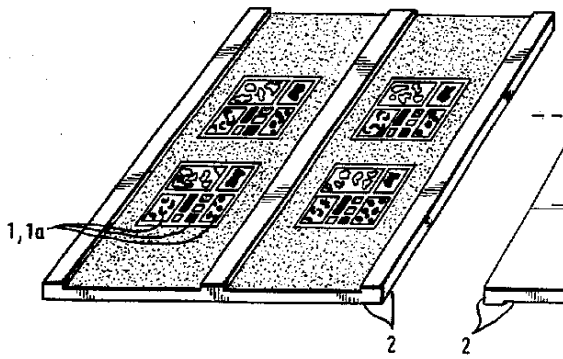


FIG. IIb

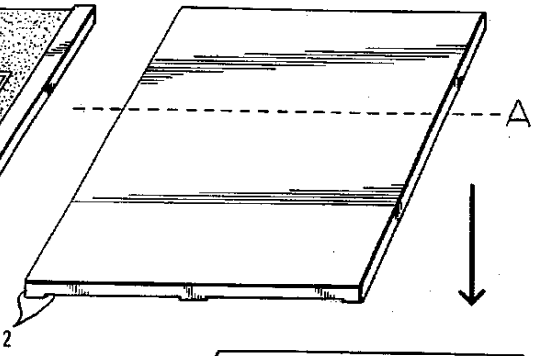


FIG. IId

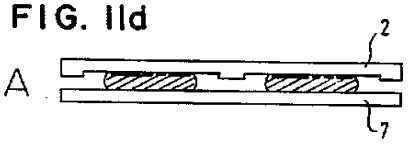


FIG. IIc

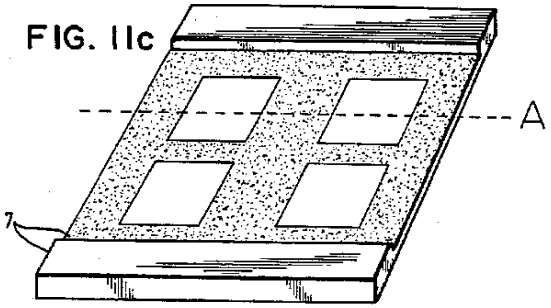


FIG. 12

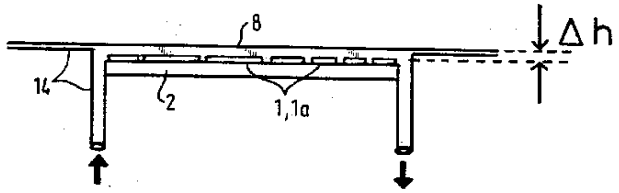


FIG. 13a

FIG. 13b

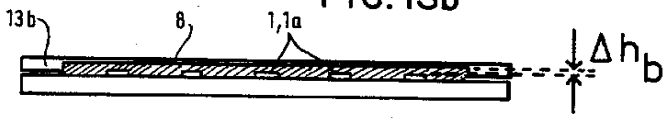


FIG. 14a

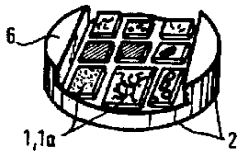


FIG. 14b

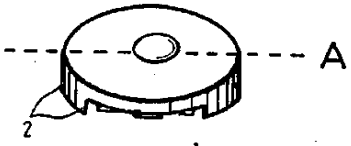


FIG. 14d

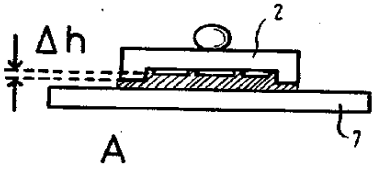
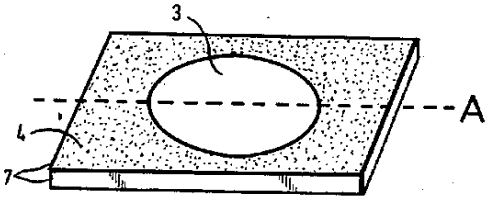


FIG. 14c



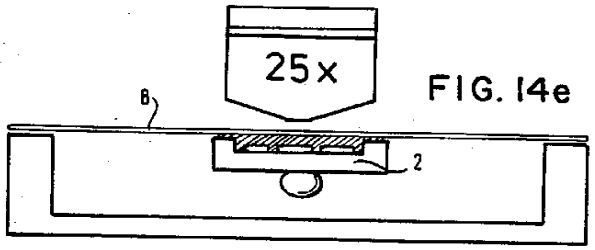


FIG. 14e

PROCESS FOR ANALYSES TO BE CARRIED OUT ON IMMOBILIZED BIOLOGICAL TISSUE

FIELD OF THE INVENTION

The present invention describes an advantageous process and equipment for testing biological material which has been immobilized and for analyzing samples employing immobilized biological material.

BACKGROUND

The prior art and effectiveness of the present invention will both be illustrated using, as an example, the immunofluorescence testing of frozen sections.

In many patients, indirect immunofluorescence testing of frozen sections makes it possible to detect antibodies against the body's own tissue. The method was introduced by Coons et al (Coons A. H., Creech H. J., Jones R.N., Proc.Soc.Exp. Biol. N.Y., 47, 1941, p. 200ff).

The method, with reference to FIG. 1, may be explained as follows. In a first stage, a frozen section of healthy tissue is placed on a glass surface and is allowed to thaw and dry. As illustrated in FIGS. 1A–1C, it is covered with the dilute serum of a patient. If the serum contains antibodies against the antigens of the tissue, they remain attached to the frozen section. Antibodies which are not directed against antigens of the frozen section do not become attached and are washed off.

In a second stage, as illustrated in FIGS. 1D and 1E, antibodies obtained from animals and labelled with a fluorescent substance are then applied to the frozen section, these second stage antibodies being directed against the (already-attached) human antibodies (fluorescentlabelled antihuman serum). The (second stage) antibodies become attached to the (first stage) antihuman antibodies fixed to the frozen section and cannot be washed off.

Accordingly, if the patient's own serum contains antibodies against the tested tissue, the fluorescence microscope is able to detect the fluorescent label bonded to the corresponding tissue structures.

Frequently, direct frozen section immunofluorescence is used to investigate the tissue of patients in order to establish whether antibodies have become attached to certain tissue structures in vivo. For this purpose, frozen sections of the tissue are made and, in a direct immunofluorescence test, are brought directly together with a fluorescence-labelled antihuman serum, the first stage of the aforementioned indirect immunofluorescence test being omitted. The antibody-containing structures fluoresce in the washed products.

Different frozen section immunofluorescence techniques for physically manipulating frozen sections are known to those skilled in the art, but each has disadvantages. The techniques and their attendant disadvantages will be reviewed as part of the background discussion which follows.

It is standard practice when using immunofluorescence tests to mount the frozen sections on standard glass slides, a single slide generally being used for each frozen section. A detailed description of the test appears e.g. in Storch (Storch, W: "Immunfluoreszenzfibel", Fischer-Verlag, Jena, 1979). However, a number of sources of error are inherent in the test, which consequently requires considerable skill and large expenditures on labor and material.

A first source of error often occurs if the drops of sera added to the frozen sections run and the frozen sections then dry out. If the frozen sections become dry during the test, the results can generally not be utilized. Thus, as a precaution, large drops are prepared and reagents are wasted.

A second source of error can occur when preparing the section for microscopy. Before carrying out microscopy, the frozen section is covered with glycerin containing phosphate buffered saline and a cover slide is placed over it and should float on the glycerin. If there is an excessive dropwise addition of glycerin, however, the cover slide generally slips with the result that the microscope is contaminated and excess glycerin must be wiped away. However, removing glycerin may exacerbate the situation if too much glycerin is removed so that, as a result of capillary forces, the cover slide is drawn firmly onto the slide and squeezes the frozen section. If the cover slide is accidentally moved very tightly against the standard slide, the frozen section will likely be destroyed.

The same type of error may occur if, during microscopy, the frozen section is to be brought into the focal plane of the objective and the cover slide is brought too close to the objective. As a result, a positive result can appear negative.

Sera from different patients can be simultaneously tested on one slide. This allows combining operating sequences so that the testing process is simplified. Several frozen sections must be placed side-by-side on the slide beforehand and it must be ensured that there is no intermixing between the sera. Such simultaneous testing is facilitated by subdividing the slide into "reaction fields" which are set off from one another by a water-repelling coating on the slide (O'Neill, P., Johnson, G.D; Ann. N.Y. Acad. Sci. 177, 446–452, 1971; U.S. Pat. No. 3,736,042; EP-OS No. 79 103 987.8) or by color rings (Räisänen, S et al, J. Clin. Pathol. 33, 95–96, 1980). The more sera that are to be investigated on a slide, the smaller the preparatory expenditure during each individual test.

However, the aforementioned "simultaneous" testing technique is not conductive to carrying out more than about 20 individual tests on the same slide, particularly because the reaction fields are subject to successive dropwise application (of sera), resulting in different incubation times for the individual analyses. Testing more than about 20 samples thus generally produces too much variation in incubation time between the first and last sections. Additionally some sections might dry out and become unusuable during the period of time fluorescence-labelled antihuman serum is being added dropwise to other sections.

When large numbers of sera have to be tested side by side, e.g. 96 sera on one slide (see FIG. 2), the methods according to Stöcker are available (EP-OS No. 79 103 987.8; DE-OS No. 3,107,964). Hydrophilic reaction fields are present in a congruent arrangement on two plates and are surrounded by a water-repelling coating. Frozen sections are placed on the reaction fields of one plate, while samples, e.g. serum dilutions or the fluorescence-labelled antihuman serum are added dropwise to the reaction fields of the other plate. Both plates are then placed in a frame in such a way that the frozen sections are immersed in the liquid samples. All the frozen sections of one plate are incubated for the same period of time and no frozen section dries out during the

test, even during the application of the fluorescence-labelled antihuman serum.

The above method for testing large numbers of sera on one plate has hitherto been adopted in immunofluorescence diagnosis only in cases where it is possible to use suspendable antigens, because they can be added dropwise to the reaction fields, e.g. toxoplasmosis or loose exciters. However, those skilled in the technology of making frozen sections appreciate how difficult it can be using hitherto known processes to place 96 frozen sections cleanly and uniformly on the reaction fields of a plate. There is a high level of waste in industrial production.

It is often necessary to seek antibodies against various antigens in a serum. Several suspendable antigens can be added dropwise side by side on a reaction field and, after drying and optionally fixing, they are jointly covered with one serum dilution drop (Wang, S.P., Excerpta Medica, Amsterdam, 273–288, 1971). If one wishes to verify antibodies against different tissues, several frozen sections may be formed into a "composite section", which is then covered with a large sample or reagent drop. For this purpose, the frozen sections for each tissue can be individually produced and different frozen sections can be juxtaposed on one reaction field.

Alternatively, several fragments of different tissues can be jointly frozen into an aqueous solution of carboxymethylcellulose, followed by the simultaneous sectioning and mounting thereof (e.g. Nairn, R.C.: "Fluorescent Protein Tracing", Churchill Livingstone Edinburgh, 1976). Only a few organ fragments can be cut together in a block, however, and the fragments must be accurately trimmed to size. Moreover, this technique requires a great deal of skill and tissue may be lost. Also, frozen sections to be fixed in different ways cannot be juxtaposed in the same "composite section".

On one hand it is frequently the case that there is only little fluorescence-labelled antihuman serum or serum to be tested available for use. The test batch must then be kept as small as possible, the prerequisite for this being small frozen sections. The tissue is cut to the desired size and account is taken of the amount of material lost.

On the other hand, in the case of testing structures distributed in a non-uniform manner in the tissue, e.g. Langerhans islands of the pancreas or glomeruli of the kidneys, large sections are required in order to be sure that the desired structures are present during microscopy. It is otherwise extremely difficult (and perhaps prohibitively so) to cut islands or glomeruli from the loose frozen section.

It is frequently the case that the available organ fragments are so small that only very few frozen sections can be obtained therefrom, and the number of sections is not sufficient for the various tests to be performed. However, the structures which are of interest are frequently represented many times on these sections. An attempt can accordingly be made to subdivide the finished loose sections, either fresh or after freeze-drying. However, the desired structures are difficult to detect on the loose section and the manipulations involved in subdividing are generally very difficult.

A particular difficulty in biochemical tests on frozen sections is that the sections frequently adhere poorly to the substrate during incubation and often partly or wholly float off. If glass slides are used as the substrate, they must consequently be very carefully cleaned prior to the application of the frozen sections (chromosulphuric acid, ethanol, acetone; Storch W., see above). In certain laboratories, the glass surface is coated with glycerin and gelatin or with chicken protein (Romeis, B: "Mikroskopische Technik", Oldenbourg-Verlag, Munich, Vienna, 1968). This process constitutes no significant improvement, however, and is ignored by many scientists. For certain tissues, such as lungs, intestinal mucosa and particularly fat-rich tissue (e.g. pancreas, adrenal medulla), there has hitherto been no reliable process for maintaining unfixed frozen sections firmly on their substrate, particularly when long incubations are employed as part of the test procedure and where thorough washing must take place.

Consequently, it is therefore conventional practice to use unfixed tissue in immunohistochemistry (Wick, G. et al: "Immunofluorescence", Medizinische Verlagsgesellschaft Marburg/Lahn, 1978). Only if the antigen is soluble in water is the frozen section fixed, e.g. as in the case of the thyroid gland which has a colloid that may be made insoluble by treatment with absolute methanol.

Numerous processes are known enabling organic material to be bonded to activated surfaces. Particular efforts are being made to immobilize enzymes, antigens and antibodies on solids (e.g. Ternynck, T., Avrameas, S., FEBS-Letters 23, 24–28, 1972; Guesdon, J. L. et al, J. Immunol. Meth. 21,59–63, 1978; DE-AS No. 2,102,514; DE-OS No. 2,740,008; DE-OS No. 2,749,317; German Pat. No. 2,905,657). A description has already been given of cutting tissue embedded in polyacrylamide and bonding the sections chemically on a surface provided with reactive groups (Hausen, P., Dreyer, C., Stain Technol, 56, 287–293, 1981). It is therein assumed that it is the polyacrylamide which binds to the surface, not the tissue. However, the fact that a frozen section can firmly join itself (as unembedded tissue) to a surface which has been activated by being chemically treated so that it contains reactive groups, which section would not otherwise adequately adhere to an untreated surface, was clearly not recognized by these authors, and the technical literature otherwise fails to report thereon.

SUMMARY OF THE INVENTION

The purpose of the invention is to provide an improved process enabling tests to be carried out on immobilized biological material, together with equipment for performing the same.

As a result of the invention, it is possible to immobilize biological material by chemically bonding it to a surface and to then rationally test it with biochemical methods or use it for rational biochemical tests. The description which follows employs immunofluorescence on frozen sections of biological tissue for purposes of illustration only. Those skilled in the art will appreciate that the invention is also suitable for numerous other applications in other fields of immunology (such as fluorescence immunoassays, radio immunoassays, enzyme immunoassays), histochemistry, microbiology, clinical chemistry and hormone chemistry.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. 1A to 1E are diagrammatic illustrations of the indirect immunofluorescence test.

FIG. 2 is a schematic illustration of a flat plate having 96 hydrophilic reaction fields 3, their surrounding area 4 being hydrophobically coated.

FIG. 3 is a schematic illustration of the rational production of a plurality of frozen section fragments 1*a* from frozen section 14 with the aid of a dividable support 1.

FIG. 4 schematically illustrates a plate 2 with a support 1*a* fixed thereto in perspective plan view (*a*), cross-section (*b*) and with a cover slide in cross-section (*c*).

FIG. 5 is a diagrammatic view which illustrates how a frozen section can be chemically coupled to a support.

FIG. 6 is a perspective view illustrating a plate 2 (*a*) having supports 1*a* fixed thereto and an associated reagent support 7 (*b*) with hydrophilic reaction fields 3 and hydrophobic surrounding area 4.

FIG. 7 is a diagrammatic illustration of an indirect immunofluorescence test according to the invention.

FIG. 8 is a perspective view illustrating a plate 2 (*a*) with 48 supports 1*a* fixed thereto; the back surface (*b*) of said plate, and the associated reagent supports 7 (*c*) and both during incubation in cross-section (*d*).

FIG. 9 shows a plate 2 (*a*) with one support 1*a* fixed thereto; the back surface (*b*) of said plate, the associated reagent support 7 (*c*) and both of them during incubation in cross-section (*d*).

FIG. 10 is a perspective view illustrating a plate 2 with a plurality of supports 1*a* having different frozen sections on a reaction field 3 ("composite section").

FIG. 11 is a perspective view illustrating a plate 2 (*a*) with four "composite sections", their back surface (*b*) of said plate, the associated reagent support 7 (*c*) and both of them during incubation in cross-section (*d*).

FIG. 12 is a sectional view illustrating a flow cell, whose base is formed by a plate 2 and to which are fixed several supports la with frozen sections (also a "composite section").

FIG. 13 is a sectional view illustrating a plate 2 with 5 supports 1*a* fixed thereto and the associated reagent supports 7 during incubation, in cross-section (*a*), the same plate with cover slide 8 (*b*).

FIG. 14 illustrates a disk-shaped "plate" 2(*a*), its back surface with knob (*b*), the associated reagent supports 7 (*c*), during an incubation in cross-section (*d*) and the plate covered with glycerin and held by capillary force against a cover slide, under the microscope (*e*).

DETAILED DISCUSSION

In the specification and claims the term "plate" is used to designate a planar substrate or support into which reaction fields, as depressions, wells, etc. are cut or otherwise placed for the purpose of receiving supports having biological material immobilized thereon. The depressions can have various geometries (circular, square, elongated, etc.) and their depth is measured from the top surface of the plate. The particular spot in the depression to which the support is fixed is a "reaction field" (as previously explained), and is the position at which serum is contacted with the frozen section or other biological material. The term "support", like the term "plate", designates a planar substrate or support. The support has much smaller dimensions than the plate, however, dimensions small enough to allow the support to fit comfortably within the "depressions" or reaction fields in the plate. Importantly, the thickness dimension of the support is such that, when combined with a section of biological material immobilized on its surface, the thickness of the support plus biological material will be equal to or less than the depth of the depression. A planar protective sheet or surface designated herein as a "cover slide" or other "facing surface" may then be placed, supported by the top surface of said plate, over the depression to protect the immobilized biological material, but with the cover slide itself at most just touching the biological material or section.

Generally the invention provides a process for carying out tests on immovable biological material with general biochemical and histochemical methods, particularly the methods of enzyme, immuno and hormone chemistry, in which one or more tests are carried out side by side on a plate and in which the biological material is largely protected against damage, wherein the biological material is initially made to adhere to the surface of one or more supports and then the supports or parts thereof are fixed to a plate together with the biological material, the surface carrying the biological material being arranged in such a way that it has no direct contact with solid objects and when this is undesired, does not dry during testing.

More specifically, the invention provides a process for protecting biological test material, comprising the steps of:

immobilizing said biological material by adhering it to the surface of at least one support; and

fixing said at least one support within at least one depression in a plate, said at least one depression having a depth equal to or greater than the combined thickness of said support and said biological material.

Following fixing the support plus biological test material (i.e. on a reaction field), a cover slide or other facing surface may be placed over the depression as protection. The facing surface, at most, lies adjacent the biological material without squeezing it. Advantageously there is a definite finite distance $\Delta h > 0$ between the biological test material and the facing surface.

The biological material is firstly placed on a support to which it is allowed to adhere. One or more supports or parts la thereof are then adhered to a plate 2 or fixed thereto with other means. Using flat plates, unless other precautions are taken, the biological material is particularly sensitive to damage or drying out because it is raised above the level of the flat plate by the support and the adhering biological material. Thus, it is not possible to carry out immunofluorescence tests by conventional methods in this manner. The cover slide which is ultimately applied rests directly on the exposed sensitive frozen sections and squeezes them.

However, in the present invention the slides are placed on the plate in such a way that the biological material is largely protected against damage, and particularly during the actual testing, such that it is not squeezed between the plate and cover slide. It is also ensured that, after the test, the frozen section does not dry out if such is undesired. According to the invention, for this purpose the supports are each fixed in depressions 5 of plate 2 as shown in FIGS. 4 and 6. The depth of depression 5 relative to the thickness of support 1*a* is such that a reliable finite distance $\Delta h \geqq 0$ exists between the biological material and the top surface of plate 2.

Instead of housing support la within depression 5, it is also possible to use other geometrical or mechanical means and embodiments, as shown in FIG. 13, to set a spacing $\Delta h \geqq 0$ between the biological material and a facing surface such as cover slide 8. If the biological material is to be assessed with the microscope or some other aid, this distance Δh must be fixed in such a way that the biological material can be placed in the focal plane of the objective, or in a plane which can be reached by the sensor of the particular aid used.

According to the invention, frozen sections can be chemically bonded to the support, in such a way that reactive chemical groups (9, see FIG. 5) are coupled to the support surface prior to the application of the frozen sections, as shown in FIG. 5. However, it is also possible to use the invention with conventionally produced frozen sections, i.e. which are not chemically bonded to the slides.

The invention has numerous advantages.

As a result of the arrangement protecting the biological material, the test results are also protected. The results are more reproducible and the percentage of unsuccessful tests can be reduced from 5–10% virtually to zero. During transportation and testing, the frozen sections are not nearly as easily damaged as when using conventional flat plates. Frozen sections can be covered with glycerin by untrained personnel, and they are not destroyed by the cover slide, even if the latter presses firmly on the plate.

In FIG. 4 the reaction field 3 is in a plane lower (by virtue of being the bottom surface in depression 5) than the area directly adjacent to the reaction field, such that the surface of a support la does not project over the plane of the surrounding area. This prevents running of the serum dilution, the fluorescence-labelled antihuman serum or the glycerin and, consequently, there is no drying out of the frozen sections.

Histological products such as thin sections of biological tissue, particularly frozen sections, or products formed from other biological materials bonded to surfaces can be produced relatively cheaply, in large number, and in high quality.

It is sometimes better not to apply the biological material directly to a plate, but instead to coat a support with it and then, in an independent second stage, to fix it with the support to a plate (plate in histochemical tests=slide). To produce supports for non-histochemical tests it is possible to coat e.g. 10,000 glass fragments (3×3×0.2 mm) in a small 250 ml volume vessel. The glass fragments can then be rapidly and simply adhered to a plate, e.g. by an automatic machine. Alternatively, the biological material may be spread onto a slide and allowed sufficient time to adhere thereto. The slide may then be comminuted into fragments and the fragments adhered to a plate.

The coated slides take up little space and are not destroyed at low temperatures. Thus, they are better suited to preservation under deep-freezing conditions than e.g. standard glass slides. Frozen section fragments may be preserved (approximately 2×2×0.2 mm—as supports, e.g. cover slide material can be used) in PVC hoses or in glass ampoules which may then be sealed and stored for many years in liquid nitrogen at −196° C., e.g. 1000 pieces in a 1 ml glass ampoule.

According to the invention, frozen sections can be easily fragmented by adhering to a support which is itself easily dividable. It normally requires considerable skill to cut an interesting or desired area of appropriate size from an individual loose frozen section. If the frozen section is stabilized by a slide, however, isolation of one or more desired areas can be carried out very easily. This technique of dividing e.g. a cover slide into fragments is illustrated in FIG. 3.

For microanalyses, 100 miniature frozen sections can be cut from a 5×5 mm frozen section adhering to a cover slide within 5 minutes.

Frozen sections of individual glomeruli can easily be separated from a frozen kidney section adhering to a cover slide. The tissue is better utilized because there is no need to use far more glomeruli than necessary for each individual test. No reagents are wasted for those parts of the frozen sections which contain no glomeruli. Thus, the invention is particularly suitable for tests in which the reagents are very expensive, as in the case of monoclonal antibodies, or in which little test material is available, as is normally the case when plasmacytoma cells have to be identified.

Unlike processes presently known to the art, the invention makes it possible to fix numerous frozen sections to a plate with numerous reaction fields, as illustrated in FIGS. 2 and 8. The inventor employs tests plates having 96 reaction fields, each reaction field having a frozen section thereon.

It is equally easy to produce "composite sections". The composition of the "diversified sections" can be varied at random between individual tests. It is possible simultaneously to test, side-by-side, tissue sections which have been pretreated in different ways, e.g. fixed and unfixed frozen sections of the thyroid gland. The frozen sections are fixed to the support before the latter is fragmented and adhered to the plate. It is therefore also possible to use fixing processes which attack the hydrophobic coating of the plate.

As a result of chemically activating the support surface, the frozen sections adhere surprisingly well and, during incubation or washing processes, there is no longer a risk of washing them away, not even if they are kept for several days in the liquid or if they are vigorously washed for a long time. Freeze-dried loose sections also bond to the activated slide in an aqueous medium. It was necessary for miniature analyses according to the invention to improve the adhesion of the frozen sections, because the latter frequently became detached from smaller slide fragments.

The following examples illustrate and explain the operation of the invention.

Example 1: Production of Activated Glass Supports

Example 1A (see FIG. 5): An adequate number of 0.2 mm thick cover slides were cleaned for 3 days in chromosulphuric acid and then adequately rinsed with distilled water. The cover slides were then immersed at ambient temperature in a 2% aminoethylaminopropyltrimethoxy silane solution in ethanol/water 1/1 (vol), washed 3 times for 1 minute in absolute ethanol and dried with compressed air. Cross-polymerization was allowed to take place overnight at 70° C., followed by a 3 hour immersion at ambient temperature in a 5% aqueous solution of freshly filtered glutaraldehyde. The slides were then adequately rinsed in distilled water and dried. By virtue of this treatment the surface of the cover slides now carried self-reacting aldehyde groups 9 which could covalently bond via free amino groups to proteins in the frozen sections. The cover slides were stored for 6 months at ambient temperature without any significant activity loss.

Example 1B: Cover slides were cleaned as in Example 1A and then immersed for 3 hours at ambient temperature in a 2% methacryloxypropyltrimethoxy silane solution in ethanol/water 1/1 (vol), dried with compressed air, kept at 70° C. for 2 hours, immersed for 12 hours in 5% acrolein, washed 3 times for 15 minutes in distilled water and dried with compressed air. The surface of the cover slides now carried self-reacting aldehyde groups. The cover slides were stored for up to 6

months before they were coated with the frozen tissue sections.

Example 1C: Cleaned cover slides were immersed for 3 hours at ambient temperature in a 2% mercaptopropyltrimethoxy silane solution in 1% acetic acid in ethanol/water 1/3 (vol), and dried with compressed air. Their surface carried now self-reacting mercaptogroups, that were able to bond mercaptogroup-bearing frozen tissue sections under oxidizing conditions.

Example 1D: Cleaned cover slides were immersed for four hours at ambient temperature in a 0.1% solution of polylysine (400 000 daltons), washed 3 times in distilled water and dried with compressed air. The surface of the cover slides now carried aminogroups which could react with components of the frozen sections via ionic bonds. The cover slides were now ready to be coated with the frozen tissue sections or to be further processed as described in Example 1E.

Example 1E: Cover slides activated according to Example 1D were immersed for 3 hours at ambient temperature in a 5% aqueous solution of freshly filtered glutaraldehyde. The slides were then rinsed in distilled water and dried. Their surface now carried aminogroups with a capacity to attach frozen tissue sections via ionic bonds, as well as self-reacting aldehyde groups.

Example 2 (FIG. 3): Production of Frozen Section Fragments

Freshly prepared 5 μm thick frozen sections **14** of human liver were placed on cover slide **1** produced according to example 1, such that almost the entire surface of one side of cover slide **1** was coated. The frozen sections were allowed to thaw and dry. A diamond tip was used to scratch fields into the cover slide in the area coated with the frozen sections, followed by subdividing the cover slide into fragments. Part of the frozen section fragments **1***a* were set aside for example 3, the remainder being placed in polyvinyl chloride hoses which were sealed and stored in liquid nitrogen.

It is easier and several times faster to produce 50 fragments as described above by fragmenting a large frozen section on a glass slide (time taken a quarter minute) and then to adhere the glass-supported fragments to a plate (5 minutes) than to cut 50 frozen sections from a small tissue portion using a cryotome, followed by placing the sections on a standard slide (30 minutes). There is also a better utilization of the tissue, because the material lost when preparing a small tissue portion is retained.

Using the invention, specifically the "fragmented support" technique described above, it is possible to seek out interesting tissue areas using a magnifier microscope, while discarding unimportant areas and areas with a poor cut quality.

The cover slide can be scratched or scored for breakage into fragments prior to applying the frozen section thereon. A frozen section can also be placed on closely juxtaposed glass fragments, followed by thawing and drying and the subsequent application of the fragments to a plate. The slide can also be made from plastics, e.g. foils made from polymethylmethacrylate with reactive groups. It is possible to punch or cut fragments from the foils, e.g. using a laser.

Example 3 (See FIGS. 6 and 7): Performing an Immunofluorescence Test

In the following test, the sera of patients were tested for antibodies against nuclei. For this purpose, and referring to FIG. 6 the frozen section fragments la produced in example 2 were stuck to reaction fields **3***a* of a plate **2**. The frozen sections were at a level which was 0.1 mm lower than the top surface of ribs **6** present on plate **2**. The thickness of the ribs was about 0.3 mm.

Referring to FIG. 7, drops **11** of diluted sera were applied to reagent support **7**. The frozen sections adhering to supports **1***a* were then immersed in the drops for 30 minutes at ambient temperature and then washed in phosphate-buffered saline PBS, firstly discretely with the aid of a second reagent support **7**, then in a beaker. This was followed for 30 minutes by a second incubation of the frozen sections with fluorescence-labelled antihuman serum **12**. After a further incubation and washing process, glycerin containing phosphate buffered saline was applied and a cover slide placed over it. The frozen sections were observed with the fluorescence microscope. The same results as with the conventional method were obtained when determining the antibodies against nuclei in 200 different sera. The work expended with the new method was 10 times less and the reagent consumption 3 times less than with the conventional method.

The same operations can be carried out using plates having a random number of reaction fields, e.g. with 1 as illustrated in FIG. 9 or with 48 as illustrated in FIG. 8.

Several supports with frozen sections of different tissues can be adhered to each reaction field ("composite section", illustrated in FIGS. **10**, **11** and **14**)—one reaction field per plate (FIGS. **10** and **14**) or several reaction fields per plate (FIG. **11**).

Supports having frozen sections can also be adhered to the bottom of a flow cell **14**, as illustrated in FIG. **12**, the top of said cell being constituted by cover slide **8** positioned a safe distance $\Delta h > 0$ above the frozen sections **1***a*, but still permitting observation with the microscope. For incubation purposes, the flow cell is simply filled with the serum dilution or with the fluorescence-labelled antihuman serum. The pH-buffered common salt solution is continuously pumped through for washing purposes. This closed arrangement has the advantage that during the test the frozen sections do not dry out as easily and there is less danger to laboratory personnel as a result of infectious sera.

FIG. **13** shows that spacers (**13***a*, **13***b*) can produce the distance Δh necessary for protecting the biological material on the surface of support **1***a*. Said spacers can be part of a reagent support **7** (FIG. 13A) or cover slide **8** (FIG. **13B**) facing plate **2**.

In FIG. **14**, a disk-like "plate" **2** is shown onto which can be adhered frozen section fragments **1***a*. This plate is made from a 0.3 mm thick circular cover slide, onto which are stuck two 0.3 mm thick strips or ribs **6**. During incubation, the "plate" floats on the serum dilution or on the fluorescence-labelled antihuman serum, which is added dropwise to reaction field **3** of reagent support **7**. For microscopy glycerine containing phosphate buffered saline is introduced between the frozen sections and the cover support and the "plate" is secured by a capillary force to cover slide **8**.

What is claimed is:

1. A process for preparing biological tissue, for storage and testing thereof comprising the steps of:
(1) immobilizing a biological tissue by adhering it to a surface of at least one dividable support having at least two surfaces;
(2) breaking or cutting said dividable support into at least one smaller support; and
(3) fixing said at least one smaller support with the immobilized biological tissue thereon onto a plate.

2. The process of claim 1 wherein said plate has at least one depression therein and said immobilized biological tissue is fixed to reaction fields in said at least one depression.

3. The process of claim 1 wherein said biological tissue is a thin histological section.

4. The process of claim 1 wherein said at least one dividable support is a glass disc.

5. The process of claim 1 wherein said biological tissue comprises frozen sections which are adhered to the surface of said support by:
coupling to said support a chemical substance having groups reactive with said frozen sections, and
chemically reacting said frozen sections with said reactive groups, thereby covalently bonding said sections to said support.

6. A process for preparing biological tissue for storage and testing thereof comprising the steps of:
(1) immobilizing a biological tissue by adhering it to a surface of at least one dividable support having at least two surfaces;
(2) breaking or cutting said biological support into at least one smaller support; and
(3) fixing said at least one smaller support with the immobilized biological tissue thereon within at least one depression in a plate, said at least one depression having a depth equal to or greater than the combined thickness or said support and said biological material.

7. The process of claim 6 wherein said at least one depression is covered with a cover slide which is supported by a surface of said plate.

8. The process of claim 6 wherein said at least one depression defines a base of a flow cell, said at least one support having biological tissue immobilized thereon being fixed to said base at a distance $\Delta h \geqq 0$ from a top surface of said cell, and wherein reagent solutions used for testing said biological tissue are caused to flow through said cell.

9. The process of claim 6 wherein said biological tissue comprises frozen sections which are adhered to the surface of said support by:
coupling to said support a chemical substance having groups reactive with said frozen sections, and
chemically reacting said frozen sections with said reactive groups, thereby covalently bonding said sections to said support.

10. A process for adhering thin sections of frozen biological tissue to a support, comprising the steps of:
coupling to a support a chemical substance having reactive groups which react with a frozen biological tissue; and
chemically reacting said frozen biological tissue with said reactive groups and thereby covalently bonding said frozen biological tissue to said support.

11. The process of claim 10, wherein:
said support is made of a material which reacts with silanes, and
said chemical substance is a silane having at least one amino group which has been reacted with at least one compound having at least two aldehyde groups.

12. The process of claim 10, wherein:
said support is made of a material which reacts with silanes, and
said chemical substance is a silane having at least one methacryl group or acryl group which can be reacted with at least one compound having at least one group which reacts with methacryl groups or acryl groups and at least one aldehyde group.

13. The process of claim 10, wherein:
said support is made of a material which reacts with silanes, and
said chemical substance is a silane having at least one mercapto group.

* * * * *

US005091318A

United States Patent [19]

Anawis et al.

[11] Patent Number: 5,091,318

[45] Date of Patent: Feb. 25, 1992

[54] **BINDING OF ALLERGENS TO A SOLID PHASE**

[75] Inventors: **Mark A. Anawis**, Grayslake; **Roger E. Lindberg**, Libertyville, both of Ill.

[73] Assignee: **Abbott Laboratories**, Abbott Park, Ill.

[21] Appl. No.: **509,255**

[22] Filed: **Apr. 13, 1990**

[51] **Int. Cl.**[5] **G01N 33/563**

[52] **U.S. Cl.** **436/513**; 436/825; 436/826; 436/530; 435/20; 435/21

[58] **Field of Search** 435/20, 21; 436/513, 436/825, 826, 530

[56] **References Cited**

U.S. PATENT DOCUMENTS

3,720,760	2/1984	Bennich et al. .	
4,031,199	6/1977	Nieschulz et al. .	
4,845,027	7/1989	Calenoff et al.	436/513

FOREIGN PATENT DOCUMENTS

0083497	7/1983	European Pat. Off. .	
0131546	1/1985	European Pat. Off. .	
0135022	3/1985	European Pat. Off. .	
0367306	5/1990	European Pat. Off. .	
33338759	10/1984	Fed. Rep. of Germany .	

OTHER PUBLICATIONS

Lundblad et al., *Chemical Reagents for Protein Modification*, vol. II, Ch. 5, 123–139.
Esen et al., *Anal. Biochem.*, 132:462 (1983).
Jahn et al., *Proc. Natl. Acad. Sci. U.S.A.*, 81:(1984).
Ishikawa et al., *J. Immunoassay* 1:385 (1980).
Conradie et al., *J. Immunol. Method* 59:289 (1983).
Inouye et al., *J. Clin. Microbiol.* 19:259 (1984).
Conroy et al., *Anal. Biochem.* 137:182 (1964).

Primary Examiner—Christine Nucker
Assistant Examiner—Jeffrey Stucker
Attorney, Agent, or Firm—Daniel R. Curry; Wean Khing Wong

[57] **ABSTRACT**

A method for producing a binding assay device composed of antigens on a cellulose nitrate, cellulose nitrate/acetate or similar solid phase is described. The method involves applying to a solid phase a small amount of an allergen composition, or a pretreated allergen composition, containing a certain concentration of allergen and drying the solution. The device is used by contacting a patient test sample to the immobilized allergen and determining whether or not the test sample contains IgE antibodies for the allergen.

51 Claims, No Drawings

BINDING OF ALLERGENS TO A SOLID PHASE

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to the binding of antigens to a solid phase material for use in a diagnostic assay. In particular, the invention increases the effective amount of antigen that can be bound to the solid phase, for example, to a thin microporous sheet of nitrocellulose, thereby making more antigenic sites available for antibody binding in an immunoassay.

2. Background of the Invention

U.S. Pat. No. 3,720,760 discloses that certain immunogenic substances, called allergens, can give rise to allergic reactions in the form of asthma, hay fever, and the like, and that the blood of a patient in whom a given allergen causes an allergic reaction usually contains low concentrations of immunoglobulins, called reagin-immunoglobulins (now usually called "IgE", which term is subsequently used herein), which are directed specifically against that allergen. The patent discloses a test for sensitivity to allergens which involves injecting given allergens into the skin of a patient; a skilled observer then assesses the degree of sensitivity to each of the allergens on the basis of the observed reaction (reddening or swelling of the skin) caused by each allergen. The patent also discloses an "in vivo" test, where the patient inhales an allergen in the form of an aerosol, and the patient is deemed to be sensitive to any allergen that causes hay fever, asthma or like symptoms.

The patent further discloses an in vitro method for determining the presence of IgE in a body fluid. The method involves binding an allergen to fine particles of a copolymer, e.g., a dextran-epichlorohydrin copolymer, by treating the particles with cyanogen bromide, and suspending the particles and an allergen in an aqueous medium. A body fluid to be tested for the presence of IgE directed against that allergen is then contacted with the allergen bound to the copolymer. The product of step (2) is then brought into contact with radiolabeled antibodies which will bind to IgE, if any, that has become bound to the allergen that is bound to the copolymer. The radiation emitted from the solids of step (3), the liquid of step (3), or both can then be measured.

The covalent coupling of antigens, including allergens, to a solid phase was used to prevent or inhibit their removal from the solid phase during the assay procedure. U.S. Pat. No. 4,597,999 describes the covalent coupling of two molecular species to one another, using cross-linking agents having at least two functional groups which are subject to independent activation. Examples of such cross-linking agents include 4-methylazidobenzidimate and N-hydroxysuccinimidyl-azidobenzoate. These cross-linking agents couple spontaneously in the dark to available amino groups, as in aminopropyl glass, aminophenyl glass and aminohexylagarose, and when activated by irradiation with light of a suitable wavelength, these agents also couple with a ligand such as a drug, digoxin, a steroid, or a protein.

U.S. Pat. No. 4,425,434 describes the use of biologically active substances to fill the pores of porous titania spheroids, porous calcium phosphate spheroids, porous zirconia spheroids or similar porous support material, and that the biologically active substance can then be immobilized in the pores by precipitation and cross-linking. The biologically active substance can be a proteinaceous substance, such as an enzyme.

It has been found, however, that the covalent coupling procedures are costly to perform and time consuming. In addition, some coupling procedures can decrease the sensitivity of the assay.

BRIEF DESCRIPTION OF THE PRESENT INVENTION

The present invention involves novel allergen compositions, using a solvent such as deionized or distilled water, containing (in milligrams of protein per milliliter, as determined by a suitable protein test): from about 0.05 to about 4.0 of *Alternaria alternata* allergen; from about 0.5 to about 50 *Aspergillus fumigatus* allergen; from about 0.8 to about 81.6 of Bermuda grass (*Cynodon dactylon*) allergen; from about 0.1 to about 6.0 of birch (*Betula nigra*) allergen; from about 0.6 to about 20.6 of cat (*Felis domesticus*) allergen; from about 0.04 to about 4.5 of mountain cedar (*Juniperus ashei*) allergen; from about 0.1 to about 20.5 of Japanese cedar (*Cryptomeria japonica*) allergen; from about 0.05 to about 10.0 of Cladosporium allergen; from about 1.3 to about 38.4 of dog (*Canis familiarus*) allergen; from about 0.7 to about 22.4 of *Dermatophagoides farinae* (*D. farinae*) allergen; from about 0.6 to about 84.2 of *D. pteronyssinus* allergen; from about 0.1 to about 10.0 of elm (Ulmus) allergen; from about 0.02 to about 0.2 of feather allergen; from about 0.2 to about 20.5 of giant ragweed (*Ambrosia trifida*) allergen; from about 0.4 to about 100 of house dust allergen; from about 0.05 to about 10.5 of June/-Kentucky bluegrass (*Poa pratensis*) allergen; from about 0.2 to about 20.5 of lamb's quarters (*Chenopodium album*) allergen; from about 0.1 to about 11.5 of maple (Acer) allergen; from about 0.3 to about 90.4 of mugwort (*Artemesia heterophylla*) allergen; from about 0.1 to about 12 of mulberry (Morus) allergen; from about 0.2 to about 25.5 of oak (Quercus) allergen; from about 0.1 to about 66.8 of olive (*Olea europea*) allergen; from about 1.0 to about 40.0 of Parietaria (*Parietaria officinalis*) allergen; from about 1.7 to about 130.4 of plantain (*Plantago lanceolata*) allergen; from about 0.1 to about 4.8 of Penicillium (*Penicillium notatum*) allergen; from about 0.05 to about 8.5 of perennial rye (*Lolium perenne*) allergen; from about 0.2 to about 20.5 of short ragweed (*Ambrosia elatior*) allergen and from about 0.05 to about 6.6 of timothy (*Phleum pratense*) allergen. Such allergen concentrations have been found optimal for the preparation of immunoassay devices for the detection of anti-IgE antibodies specific for the allergens.

The present invention also involves devices for detecting the presence or amount of IgE in a test sample. The assay devices include a solid phase and an allergen immobilized upon the solid phase, wherein the allergen is typically applied as one of the above allergen compositions. In certain assay devices, the allergen composition is combined with a pretreatment substance such a denaturant, organic solvent, crosslinking agent or concentrated salt solution. It has been unexpectedly found that such allergen pretreatment can enhance allergen immobilization upon the solid phase as well as increase the effective amount of allergen that can be immobilized upon the solid phase. The reaction or binding area of the solid phase can be optionally modified by the addition of a protein blocking reagent. Suitable blocking reagents include equine serum albumin, bovine serum albumin, fish gelatin and casein.

In addition, the present invention describes allergen compositions containing a solvent, an allergen solubilized in the solvent, thereby forming an allergen solution, and a pretreatment substance chosen from denaturants, organic solvents, crosslinking agents or concentrated salt solutions, wherein the allergen solution is combined with the pretreatment substance, and wherein the resultant composition is used for the in vitro detection of the presence or amount of IgE in a test sample. In vitro detection methods can involve: providing a solid phase prepared by applying the novel allergen compositions or pretreated allergen compositions to the solid phase; contacting the sample to be tested to that solid phase, thereby immobilizing allergen-specific IgE antibody from the sample upon the solid phase by forming allergen/antibody complexes; and detecting that immobilized allergen-specific antibody to determine the presence or amount of the antibody in the test sample. Generally, the solid phase is contacted with an indicator reagent to determine the presence or amount of IgE in the test sample, wherein the indicator reagent includes a label conjugated to a binding member that is specific for either the allergen, IgE or an ancillary specific binding member. The label that is selected is not critical to the present invention and is typically chosen from chromogens, catalysts, fluorescent compounds, chemiluminescent compounds, radioactive isotopes, colloidal metallic particles, colloidal selenium particles, dye particles, enzymes, substrates, organic polymer latex particles and liposomes or other vesicles containing signal producing components. The present invention also includes assay kits containing the allergen or allergens of interest immobilized upon the solid phase and a suitable indicator reagent. Optionally, the kit can include assay buffers and wash reagents.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention is based upon the discovery that an allergen solution can be used to bind an allergen to a solid phase material without the need for covalent linkages. A solid phase so prepared can then be used in an in vitro diagnostic assay for IgE. Suitable solid phase materials include cellulose nitrate or a mixed ester cellulose. In addition, it has been discovered that certain allergen concentrations are optimum insofar as the sensitivity of the assay is concerned.

The invention is also based upon the discovery that many allergens can be pretreated to improve their adherence to the solid phase material. The allergen pretreatment methods of the present invention serve to enhance the binding of the allergen to the solid phase throughout the assay. The allergen pretreatment compositions and methods were also unexpectedly found to increase the amount of allergen which can be bound to the solid phase thereby enabling the binding of allergen in an amount that is optimal for the assay.

The present invention involves novel allergen compositions for the preparation of solid phase devices used in binding assays. The allergen compositions have been unexpectedly found to enhance the binding of the allergen to the solid phase material. As a result, greater amounts of antigen may be immobilized upon the solid phase, thereby providing more antigenic sites for binding antibody during the assay.

The present invention also involves the pretreatment of certain allergen compositions with substances such as denaturants, organic solvents, crosslinking agents and concentrated salt solutions. Pretreatment of an allergen composition with one or more of these substances was unexpectedly found to enhance the adherence of the allergen to a solid phase throughout the assay procedure which may include multiple washing steps or other manipulations which could otherwise dislodge the allergen from the solid phase. In addition, the pretreatment of the allergen improves their binding performance at elevated temperatures often used in binding assays.

Suitable denaturants include, but are not limited to: acids such as hydrochloric acid (HCl) and acetic acid. Organic solvents, such as tetrahydrofuran, are suitable for allergen pretreatment. Concentrated salt solutions, such as concentrated solutions of sodium chloride (NaCl), are also suitable for allergen pretreatment according to the present invention. Suitable cross-linking agents for the pretreatment of allergens include, but are not limited to: formaldehyde, glutaraldehyde and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC).

Allergen compositions combined with such pretreatment substances are then used in the production of novel solid phase assay devices. The allergen compositions or pretreated allergen compositions are applied to a solid phase material upon which the allergen composition is dried and thereby immobilized. The solid phase devices can then be used in binding assays which include, but are not limited to, competitive assays, sandwich assays and indirect assays, and include both forward and reverse assay formats.

In a preferred embodiment of the present invention, the allergen of interest is immobilized upon a solid phase material made of nitrocellulose or a nitrocellulose derivative or compound, such as cellulose acetate/nitrate mixed ester cellulose. The maximum binding capacity of nitrocellulose for the protein bovine serum albumin is about 140 $\mu g/cm^2$. This binding capacity value is converted according to the desired size of the solid phase reaction or binding area of the present invention, and a value of 2.2 mg/ml is obtained. This concentration is used as the starting protein concentration for all allergens, but the optimum allergen concentration may be above or below this value. Different concentrations of allergen solutions are pretreated, immobilized on nitrocellulose and tested with a positive test sample, as described in the specific examples which follow. The allergen concentration is adjusted such that when concentration is plotted against signal a parabolic curve is obtained, and the optimum allergen concentration can be determined from the maximum detected signal.

The allergen protein concentrations which were tested ranged from about 0.05 milligrams of allergen per milliliter of solvent, prior to pretreatment, to about 170 mg/mL. The most effective concentration ranges for each of the allergens tested are presented in the specific examples which follow.

The invention will be more fully understood from the following examples, which constitute the best modes presently contemplated by the inventors. It is to be understood, however, that the examples are presented solely for the purpose of illustration, and are not to be construed as limiting.

Before proceeding with the description of the specific embodiments of the present invention, a number of terms will be defined. All allergen contents herein refer to the protein content of the allergen solutions, determined using a suitable protein test such as Coomasie blue or Ninhydrin as are well-known in the art.

The term "analyte" refers to the substance to be detected in or separated from test sample. The analyte can be any substance for which there exists a naturally occurring specific binding member or for which a specific binding member can be prepared. In addition, the analyte may bind to more than one specific binding member. "Analyte" also includes any antigenic substances, haptens, antibodies, and combinations thereof. In the present invention, the main analytes to be detected or measured are IgE antibodies.

The term "test sample" refers to virtually any liquid sample. The test sample can be derived from any desired source, such as a physiological fluid, for example, blood, saliva, ocular lens fluid, cerebral spinal fluid, sweat, urine, milk, ascites fluid, mucous, synovial fluid, peritoneal fluid, amniotic fluid or the like. The liquid test sample can be pretreated prior to use, such as preparing plasma from blood, diluting viscous liquids, or the like; methods of treatment can also involve separation, filtration, distillation, concentration, inactivation of interfering components, and the addition of reagents. In addition, a solid can be used once it is modified to form a liquid medium.

The term "specific binding member" refers to a member of a specific binding pair, i.e., two different molecules wherein one of the molecules through chemical or physical means specifically binds to the second molecule. In addition to antigen and antibody specific binding pairs such as the allergen and antibody pair, other specific binding pairs include, biotin and avidin, carbohydrates and lectins, complementary nucleotide sequences, complementary peptide sequences, effector and receptor molecules, enzyme cofactors and enzymes, enzyme inhibitors and enzymes, a peptide sequence and an antibody specific for the sequence protein, polymeric acids and bases, dyes and protein binders, peptides and specific protein binders (e.g., ribonuclease, S-peptide and ribonuclease S-protein), and the like. Furthermore, specific binding pairs can include members that are analogs of the original specific binding member, for example an analyte-analog. If the specific binding member is an immunoreactant it can be, for example, an antibody, antigen, hapten, or complex thereof. If an antibody is used, it can be a monoclonal or polyclonal antibody, a recombinant protein or antibody, a mixture or mixtures or a fragment or fragments thereof, as well as a mixture of an antibody and other specific binding members. The details of the preparation of such antibodies and their suitability for use as specific binding members are well-known to those skilled-in-the-art.

An "indicator reagent", as used herein, refers to a label attached to a specific binding member. The indicator reagent produces a detectable signal at a level relative to the amount of an analyte in the test sample. Generally, the indicator reagent is detected or measured after it is captured on the solid phase material, but the unbound indicator reagent can also be measured to determine the result of an assay. The specific binding member component of the indicator reagent enables the indirect binding of the label to the analyte, to an ancillary specific binding member, to the capture reagent or to a complex thereof.

The term "label" refers to any substance which is attached to a specific binding member and which is capable of producing a signal that is detectable by visual or instrumental means. Suitable labels for use in the present invention can include chromogens; catalysts; fluorescent compounds; chemiluminescent compounds; radioactive isotopes; direct visual labels including colloidal metallic and non-metallic particles, dye particles, enzymes or substrates, or organic polymer latex particles; liposomes or other vesicles containing signal producing substances; and the like.

Many enzymes suitable for use as labels are disclosed in U.S. Pat. No. 4,275,149, columns 19–23, herein incorporated by reference. For example, an enzyme/substrate signal producing system useful in the present invention is the enzyme alkaline phosphatase wherein the substrate used can be 5-bromo-4-chloro-3-indolyl phosphate or a derivative or analog thereof. If horseradish peroxidase is used, o-Phenylenediamine or 4-chloronaphthol is added as an enzyme substrate to form a colored product which can be detected and/or measured visually or instrumentally.

In an alternative signal producing system, the label can be a fluorescent compound where no enzymatic manipulation of the label is required to produce a detectable signal. Fluorescent molecules such as fluorescein, phycobiliprotein, rhodamine and their derivatives and analogs are suitable for use as labels in this system.

An especially preferred class of labels includes the visually detectable, colored particles which enable a direct colored readout of the presence or concentration of the analyte in the test sample without the need for using additional signal producing reagents. Materials for use as such particles include colloidal metals, such as gold, and dye particles as disclosed in U.S. Pat. Nos. 4,313,734 and 4,373,932. The preparation and use of non-metallic colloids, such as colloidal selenium particles, are disclosed in co-owned and copending U.S. patent application Ser. No. 072,084, filed July 9, 1987, which is incorporated by reference herein in its entirety. Organic polymer latex particles for use as labels are disclosed in co-owned and copending U.S. patent application Ser. No. 248,858, filed Sept. 23, 1988, which is incorporated by reference herein in its entirety.

A variety of different indicator reagents can be formed by varying either the label or the specific binding member; it will be appreciated by one skilled-in-the-art that the choice involves consideration of the analyte to be detected and the desired means of detection. The selection of a particular label is not critical, so long as the label is capable of generating a detectable signal either by itself or in conjunction with one or more additional signal producing components. The details of the preparation of such label/specific binding member conjugates are well-known to those skilled-in-the-art.

The term "signal producing component" refers to any substance capable of reacting with another assay reagent or the analyte to produce a reaction product or signal that indicates the presence of the analyte and that is detectable by visual or instrumental means. "Signal production system", as used herein, refers to the group of assay reagents that are needed to produce the desired reaction product or signal. For example, one or more signal producing components can be used to react with a label and generate the detectable signal, i.e., when the label is an enzyme, amplification of the detectable signal is obtained by reacting the enzyme with one or more substrates or additional enzymes to produce a detectable reaction product.

The term "capture reagent" refers to a capture binding member which is attached to a solid phase material to enable the separation of the analyte or indicator reagent, that is bound thereto, from unbound analyte and assay reagents. Typically, the attachment of the capture

binding member to the solid phase material is substantially irreversible.

In forming a capture reagent to be used in an assay, once the capture binding member, e.g., allergen, is immobilized upon the solid phase, the remaining surface area of the solid phase is generally blocked with a suitable inactivating solution, such as bovine or equine serum albumin, casein or other proteinaceous material, to prevent non-specific binding of protein to the solid phase when the reaction mixture containing a specific binding member is contacted to the solid phase. The solid phase is then washed with an appropriate solution to remove any excess blocking solution and/or unbound capture binding member.

Once complex formation occurs between the assay components, the solid phase can be used as a separation mechanism. For example, the reaction mixture can be contacted to the capture reagent, and the solid phase material retains the newly formed reaction complex(es).

Assay devices can have many configurations, several of which are dependent upon the material chosen for the solid phase. The term "solid phase material" refers to any suitable chromatographic, bibulous, porous or capillary material or other conventional solid material, well-known to those skilled-in-the-art for use in immobilizing specific binding members. Solid phase materials can include fiberglass, nylon or cellulose or derivatives thereof, such as cellulose nitrate or a cellulose acetate/-cellulose nitrate mixed ester cellulose. The solid phase, however, is not limited to porous materials. The solid phase material can also include, without limitation, polymeric or glass beads, microparticles, tubes, sheets, plates, slides, magnetic beads, a microtitre plate with one or more reaction wells or a glass or plastic test tube, or the like.

Natural, synthetic or naturally occurring materials that are synthetically modified, can be used as a solid phase material including polysaccharides, e.g., cellulose materials including paper, cellulose and cellulose derivatives such as cellulose acetate, nitrocellulose and cellulose acetate/nitrate mixed ester cellulose; silica; fiberglass; inorganic materials such as deactivated alumina, diatomaceous earth or other inorganic finely divided material uniformly dispersed in a porous polymer matrix, with polymers such as vinyl chloride, vinyl chloride-propylene copolymer, and vinyl chloride-vinyl acetate copolymer; cloth, both naturally occurring (e.g., cotton) and synthetic (e.g., nylon); porous gels such as silica gel, agarose, dextran and gelatin; polymeric films such as polyacrylamide; magnetic particles; microtitre plates; polystyrene tubes; protein binding membranes; agarose; Sephadex® (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.); Trisacryl (Pointet-Girard, France); silicon particles; porous fibrous matrixes; and the like. The solid phase material should have a reasonable inherent strength or strength can be provided by means of a support, and it should not interfere with the production of a detectable signal.

Optionally, the specific binding member of the capture reagent can be affixed to particles, e.g., microparticles. These microparticles can serve as the solid phase material and be retained in a column, suspended in a mixture of soluble reagents and test sample, or retained and immobilized by another solid phase base material. By "retained and immobilized" is meant that the microparticles, associated with the solid phase base material, are not capable of substantial movement to positions elsewhere within that material. The microparticles can be selected by one skilled-in-the-art from any suitable type of particulate material including those composed of polystyrene, polymethylacrylate, polypropylene, polytetrafluoroethylene, polyacrylonitrile, polycarbonate or similar materials. The size of the microparticles is not critical, although it is preferred that the average diameter be smaller than the average pore size of the solid phase base material if such is used.

The term "ancillary specific binding member" refers to a specific binding member used in addition to the specific binding members of the capture reagent and the indicator reagent. One or more ancillary specific binding members can be used in an assay. For example, an ancillary specific binding member can be used in an assay where the specific binding member of the indicator reagent is capable of binding the ancillary specific binding member which is in turn capable of binding the analyte.

The present invention is concerned with immunoassays. Therefore, the following discussion of immunoassays and definitions of terms often used with respect to immunoassays are set forth to facilitate the understanding of the disclosure and claims hereof.

In accordance with one method of the present invention, a sandwich assay can be performed wherein a capture reagent can include an allergen which has been bound to a solid phase material. The capture reagent is contacted with a test sample, suspected of containing the analyte, and an indicator reagent containing an analyte-specific binding member conjugated to a label. The reagents can be contacted to the sample simultaneously or added sequentially. A binding reaction results in the formation of a capture reagent/analyte/indicator reagent complex. The assay may also involve a washing step to separate the resultant complex from the excess reagents and test sample. Either the unreacted indicator reagent or the complex retained upon the solid phase is then observed to detect or measure the amount of label associated therewith. If analyte is present in the sample, then label will be present on the solid phase material. The amount of label on the solid phase is proportional to the amount of analyte in the sample.

The present invention also can be used to conduct a competitive assay. In one example of a competitive configuration, the capture reagent again includes a specific binding member (allergen) which has been attached to a solid phase material. The capture reagent is contacted with both test sample and an indicator reagent that includes an analyte or analyte analog which has been labeled with a signal generating compound. The indicator reagent and analyte then compete in binding to the capture reagent. The competitive binding reaction results in the formation of capture reagent-/analyte complexes or capture reagent/indicator reagent complexes. The capture reagent/indicator reagent complexes can be detected via the label of the indicator reagent. In the competitive assay, the amount of label that becomes associated with the solid phase is inversely proportional to the amount of analyte in the sample.

The present invention can also be used in indirect immunoassays involving one or more ancillary specific binding members. For example, an indirect sandwich immunoassay with the formation of a capture reagent-/analyte/anti-analyte antibody/indicator reagent complex can be performed, wherein the indicator reagent is a specific binding partner for the ancillary specific binding member which is specific for the analyte. The pres-

ent invention can also be used in forward and reverse immunoassay protocols

EXAMPLES

Example 1

In this experiment, *Alternaria alternata* allergen was pretreated for binding to a solid phase material. A 37% aqueous formaldehyde solution (12.5 μL) was mixed with 100 microliters of a solution of *Alternaria alternata* (28.8 μg/mL) in deionized water. The amount of formaldehyde effective for pretreatment was found to range from about 10 μL to about 20 μL when the 37% aqueous formaldehyde solution was used. The resulting mixture was incubated at 4° C. for about 10 hours, and the incubated composition was allowed to stand for 30 to 60 minutes at about 20° C. The mixture was then centrifuged, and the resultant supernatant, a pretreated *Alternaria alternata* allergen composition, was decanted. The pretreated composition was poured onto a disc of microporous cellulose nitrate (about 140 μm thick and about 3 mm in diameter) and allowed to dry. The allergen was thereby immobilized upon the solid phase material. The remaining surface of the disc was then blocked with a ten percent horse serum solution.

The solid phase bound allergen, or *Alternaria alternata* capture reagent, was then used in an enzyme immunoassay ("EIA"). The EIA method included the following steps. The sample to be tested (e.g., serum) was contacted to the capture reagent, thereby immobilizing allergen-specific IgE antibodies upon the solid phase. Optionally, the antibody immobilization step was followed by a wash step to remove unbound sample. The capture reagent was then contacted to an enzyme-labeled anti-IgE antibody (indicator reagent) which bound to that IgE from the sample, if any, which had bound to the solid phase. The solid phase was then washed to remove unbound indicator reagent. The solid phase was contacted to an enzyme substrate signal producing component such that the enzyme component of the complexed indicator reagent would react with the substrate to produce a detectable signal. Prior to detection, the solid phase may undergo a third washing to remove unbound substrate. The signal which was detected was directly related to the amount of allergen-specific IgE in the test sample.

In one EIA procedure, the enzyme label was alkaline phosphatase, the substrate was 5-bromo-4-chloro-3-indolylphosphate and the detection or measurement step was performed with a reflectance spectrophotometer. The disc turned dark blue upon the addition of substrate to the solid phase, i.e., a positive assay result, when the serum sample contained IgE antibody specific to *Alternaria alternata*. The assay procedure was repeated using serum from different patients, and the results were found to correlate with the results obtained for the same serum samples using alternate tests, such as a radio-allergo-sorbent test (RAST) or a skin prick test as are well-known in the art.

Example 2

In this experiment, the nitrocellulose disc used as the solid phase was one of many discs on a laminate composed of a mylar sheet to which a sheet of nitrocellulose had been glued. A circular shape was embossed onto the nitrocellulose sheet to form each of the discs. The micropores in the nitrocellulose sheet had diameters of about 450 nanometers. Each individual disc had a separate allergen attached thereto. Thus, the device could be used to detect the presence of antibodies to multiple allergens.

Example 3

The procedure of Example 1 was repeated using 100 microliter portions of solutions containing birch allergen (137 μg) or dog allergen (280 μg) which were mixed with a 37% aqueous formaldehyde solution (12.5 μL) and incubated thereby forming pretreated allergen compositions. The amount of formaldehyde effective for pretreatment was found to range from about 10 μL to about 15 μL when the 37% aqueous formaldehyde solution was used. The compositions were used substantially in accordance with the procedures described in Examples 1 and 2 to produce devices which were then used to test serum samples. The assay results were found to correlate with the results of testing the same serum samples by other means: the disc turned dark blue when the serum sample contained IgE specific for the allergen immobilized upon the solid phase.

Example 4

Tetrahydrofuran (25 μL) was mixed with 100 microliters of a solution containing Bermuda grass allergen (510 μg) in deionized water. The amount of tetrahydrofuran effective for pretreatment was found to range from about 10 μL to about 50 μL. The resulting mixture was incubated at 4° C. for about 10 hours, and the incubated composition was allowed to stand at about 20° C. for 30 to 60 minutes. The solution was then centrifuged, and the resultant supernatant, a pretreated Bermuda grass allergen composition, was decanted.

The procedure was repeated using 100 microliter-portions of solutions which contained Japanese cedar allergen (150 μg), June/Kentucky blue grass allergen (545 μg), perennial rye allergen (433 μg) or timothy allergen (43 μg) in deionized water. The pretreated allergen compositions were used to produce solid phase discs and were used in immunoassays substantially in accordance with the procedure described in Example 1. The assay results were found to correlate with the results of testing the same serum samples by other means: the disc turned dark blue when the serum sample contained IgE specific for the allergen immobilized upon the solid phase.

Example 5

A 37 percent aqueous formaldehyde solution (15.6 μg) was mixed with 100 microliters of a solution containing mountain cedar allergen (733 μg) in deionized water. The resulting mixture was incubated at about 20° C. for approximately 30 minutes. Tetrahydrofuran (28.7 μL) was then mixed with the incubated solution. The amount of formaldehyde effective for pretreatment was found to range from about 10 μL to about 20 μL, and the amount of tetrahydrofuran was found to range from about 10 μL to about 50 μL. The mixture was incubated for about 10 hours at 4° C. and was allowed to stand at about 20° C. for 30 to 60 minutes. The mixture was then centrifuged, and the resultant supernatant, a pretreated cedar allergen composition, was decanted.

This allergen pretreatment procedure was repeated, using 100 microliter-portions of solutions containing oak allergen (729 μg) or olive allergen (1670 μg), in deionized water, in place of the mountain cedar allergen. The pretreated allergen compositions were then used to produce immunoassay devices substantially in

accordance with the procedures described in Examples 1 and 2. The EIA results were found to correlate with the results of testing of the same serum samples by other means: the disc turned dark blue when the serum sample contained IgE specific for the allergen immobilized upon the solid phase.

Example 6

Aqueous NaCl (5M, 12 μL) was mixed with 100 microliters of a solution containing Cladosporium (960 μg) in deionized water. The resulting mixture was incubated at about 4° C. for about 10 hours, and the incubated composition was then allowed to stand at about 20° C. for 30 to 60 minutes. The mixture was then centrifuged, and the resultant supernatant, a pretreated Cladosporium allergen composition, was decanted. Depending upon the molar value of the concentrated salt solution used, which value ranged from about 0.5M to about 10M, the amount of aqueous NaCl effective for pretreatment ranged from about 10 μL to about 20 μL.

The procedure was repeated using 100 microliters of a solution containing feather allergen (7 μg) in deionized water. The pretreated allergen compositions were then used to produce assay devices and were used in immunoassays substantially in accordance with the protocol described in Example 1. The assay results using the compositions and devices of the present invention were found to correlate with the results of testing the same serum samples by other means: the disc turned dark blue when the serum contacted thereto contained IgE specific for the allergen immobilized upon the solid phase.

Example 7

An aqueous solution of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC, 10 μL at 50 mg/mL) was mixed with 100 microliters of a solution containing D. farinae (280 μg) in deionized water. The amount of EDAC effective for the first stage of pretreatment ranged from about 5.0 μL to about 15 μL. The resulting mixture was incubated at about 22° C. for about 15 minutes. A two microliter portion of a solution containing sodium borohydride (20 mg/mL, $NaBH_4$) in 10 μM phosphate buffered saline (pH 7) was mixed with the incubated solution, and the mixture was further incubated at about 4° C. for 10 hours. The amount of $NaBH_4$ effective for the second stage of pretreatment ranged from about 1.0 μL to about 5.0 μL. The mixture was then allowed to stand at about 20° C. for approximately 30 to 60 minutes. The mixture was centrifuged, and the resultant supernatant, a pretreated D. farinae allergen composition, was decanted.

The procedure was repeated using 100 microliters of a solution containing *D. pteronyssinus* (263 μg) in deionized water. The pretreated allergen compositions were used substantially in accordance with the procedures described in Example 1 to produce treated discs for immunoassays. The EIA results were found to correlate with the results of testing the same serum samples by other means: the disc turned dark blue when the serum sample contacted thereto contained IgE specific for the allergen immobilized upon the solid phase.

Example 8

One hundred percent acetic acid (12.5 μL, with effective amounts ranging from about 5.0 μL to about 30 μL) was mixed with 100 microliters of a solution containing lamb's quarters allergen (1176 μg) in deionized water. The resultant mixture was incubated at about 22° C. for approximately five minutes, after which time 6N aqueous NaOH was added to adjust the pH to 7. The neutralized solution was incubated at about 4° C. for 10 hours, and was then allowed to stand at about 20° C. for 30 to 60 minutes. The mixture was then centrifuged, and the resultant supernatant, a pretreated lamb's quarters allergen composition, was decanted.

The procedure was repeated using 100 microliters of a solution containing mulberry allergen (40 μg) in deionized water. The pretreated allergen compositions were used to produce treated discs for enzyme immunoassays substantially in accordance with the procedures described in Example 1. The assay results were found to correlate with the results of testing the same serum samples by other means: the disc turned dark blue when the serum sample contacted thereto contained IgE specific for the allergen immobilized upon the solid phase.

Example 9

A solution of 6N aqueous HCl (24 μL, with effective amounts ranging from about 6.0 μL to about 30 μL) was mixed with 100 microliters of a solution containing Penicillium (120 μg). The resulting mixture was incubated for approximately five minutes at about 20° C., after which time 6N aqueous NaOH was added to adjust the pH to 7. The neutralized solution was incubated at 4° C. for 10 hours and was then allowed to stand at about 20° C. for 30 to 60 minutes. The mixture was then centrifuged, and the resultant supernatant, a pretreated Penicillium allergen composition, was decanted.

The allergen pretreatment procedure was repeated with 100 microliters of a solution containing Parietaria allergen (400 μg) in deionized water. The solutions were then used substantially in accordance with the procedures described in Example 1 to produce discs and to test serum samples in an EIA. The assay results were found to correlate with the results of testing the same serum samples by other means: the disc turned dark blue when the serum contacted thereto contained IgE specific for the allergen immobilized upon the solid phase.

Example 10

Untreated allergen compositions included from about 0.5 to about 50 Aspergillus allergen; from about 0.6 to about 20.6 of cat allergen; from about 0.1 to about 10.0 of elm allergen; from about 0.4 to about 100 of house dust allergen; from about 0.1 to about 11.5 of maple allergen; from about 0.3 to about 90.4 of mugwort allergen and from about 1.7 to about 130.4 of plantain allergen in deionized water.

The solutions were then used substantially in accordance with the procedures described in Example 1 to produce discs and to test serum samples in an EIA. The assay results were found to correlate with the results of testing the same serum samples by other means: the disc turned dark blue when the serum sample contained IgE specific for the allergen immobilized upon the solid phase.

Example 11

Pretreated allergen compositions, which were produced as described in Examples 1, and 3 through 9, and which differed from one another with respect to allergen content, were used to test for IgE in a series of serum samples. Upper and lower allergen concentration limits were set by classifying a pretreated allergen composition as either "too dilute" if that composition failed

to produce a maximum positive IgE test result with a serum sample which had tested positive with a more concentrated allergen solution, or "too concentrated" if the composition failed to produce a maximum positive IgE test result with a serum sample which had tested positive with a less concentrated allergen solution. The allergen concentrations tested ranged from about 0.05 milligrams of allergen per milliliter of water, prior to pretreatment, to about 170 milligrams/milliliter. The test results are presented in Table 1 and illustrate the most effective concentration ranges for each of the allergens tested.

TABLE 1

Allergen	Effective concentration range (protein content in solution)
Alternaria alternata allergen	from 0.05 to 4.0 mg/mL
Aspergillus fumigatus allergen	from 0.5 to 50.0 mg/mL
Bermuda grass allergen	from 0.8 to 81.6 mg/mL
birch allergen	from 0.1 to 6.0 mg/mL
mountain cedar allergen	from 0.04 to 4.5 mg/mL
Japanese cedar allergen	from 0.1 to 20.5 mg/mL
Cladosporium allergen	from 0.05 to 38.4 mg/mL
cat allergen	from 0.6 to 20.6 mg/mL
dog allergen	from 1.3 to 38.4 mg/mL
D. farinae allergen	from 0.7 to 22.4 mg/mL
D. pteronyssinus allergen	from 0.6 to 84.2 mg/mL
elm allergen	from 0.1 to 146.0 mg/mL
feather allergen	from 0.02 to 0.2 mg/mL
giant ragweed allergen	from 0.2 to 148.2 mg/mL
house dust allergen	from 0.4 to 100 mg/mL
June/Kentucky bluegrass allergen	from 0.05 to 21.8 mg/mL
lamb's quarters allergen	from 0.2 to 47.0 mg/mL
maple allergen	from 0.1 to 166.3 mg/mL
mugwort allergen	from 0.3 to 90.4 mg/mL
mulberry allergen	from 0.1 to 12 mg/mL
oak allergen	from 0.2 to 29.2 mg/mL
olive allergen	from 0.1 to 66.8 mg/mL
Parietaria allergen	from 1.0 to 40.0 mg/mL
plantain allergen	from 1.7 to 130.4 mg/mL
Penicillium allergen	from 0.1 to 4.8 mg/mL
perennial rye allergen	from 0.05 to 17.3 mg/mL
short ragweed allergen	from 0.2 to 151.6 mg/mL
timothy allergen	from 0.05 to 6.6 mg/mL

In this manner, the optimum concentration of allergen was determined for the production of solid phase assay devices.

It will be appreciated by one skilled-in-the-art that the concepts of the present invention are equally applicable to many different allergens (specific binding members), solid phase materials and immunoassay protocols. It will also be appreciated that the selection of any given label, ancillary binding member or solid phase material is generally not critical to the present invention. The materials are selected to optimize the results provided by the chosen assay configuration. The embodiments described herein are intended as examples rather than as limitations. Thus, the description of the invention is not intended to limit the invention to the particular embodiments described in detail, but it is intended to encompass all equivalents and subject matter within the spirit and scope of the invention as described above and as set forth in the following claims.

What is claimed is:

1. A device, for detecting the presence or amount of IgE in a test sample, comprising:

a) a solid phase comprising a material selected from the group consisting of: nitrocellulose, nitrocellulose derivatives, nitrocellulose compounds, or combinations thereof, and

b) at least one allergen immobilized upon said solid phase, wherein said allergen was applied as an allergen composition and

wherein said allergen composition is formed from the combination of said allergen with a pretreatment substance selected from the group consisting of: denaturants excluding organic solvents and concentrated salt solutions; organic solvents; crosslinking agents; concentrated salt solutions; and combinations thereof.

2. The device according to claim 1, wherein said pretreatment substance is hydrochloric acid or acetic acid.

3. The device according to claim 1, wherein said pretreatment substance is tetrahydrofuran.

4. The device according to claim 1, wherein said pretreatment substance is a concentrated sodium chloride solution.

5. The device according to claim 1, wherein said pretreatment substance is formaldehyde, glutaraldehyde or 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide.

6. The device according to claim 1, where said solid phase is selected from the group consisting of cellulose, cellulose derivatives, silica, fiberglass, a porous polymer matrix, porous gels, polymeric films, agarose and porous fibrous matrixes.

7. The device according to claim 1, where said solid phase is selected from the group consisting of cellulose acetate, nitrocellulose and cellulose acetate/nitrate mixed ester cellulose.

8. The device according to claim 1, further comprising a protein blocking reagent on said solid phase.

9. A method for producing a device according to claim 1, comprising the steps of:

a) forming the allergen composition by pretreating the allergen with a substance selected from the group consisting of: denaturants excluding organic solvents and concentrated salt solutions; organic solvents; crosslinking agents; concentrated salt solutions; and combinations thereof

b) applying said allergen composition to said solid phase, and

c) drying said allergen composition on said solid phase, thereby immobilizing said allergen upon said solid phase.

10. The method according to claim 9, further comprising the step of applying a protein blocking reagent to said solid phase.

11. The method according to claim 10, wherein said protein blocking reagent is selected from the group consisting of equine serum albumin, bovine serum albumin, fish gelatin and casein.

12. The method according to claim 11, wherein said pretreatment substance is hydrochloric acid or acetic acid.

13. The method according to claim 11, wherein said pretreatment substance is tetrahydrofuran.

14. The method according to claim 11, wherein said pretreatment substance is a concentrated sodium chloride solution.

15. The method according to claim 11, wherein said pretreatment substance is formaldehyde, glutaraldehyde or 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide.

16. The method according to claim 9, where said solid phase is selected from the group consisting of cellulose, cellulose derivatives, silica, fiberglass, a po-

rous polymer matrix, porous gels, polymeric films, agarose and porous fibrous matrixes.

17. The method according to claim 9, where said solid phase is selected from the group consisting of cellulose acetate, nitrocellulose and cellulose acetate/-nitrate mixed ester cellulose.

18. The device of claim 1, wherein the allergen composition contains, on the basis of allergen protein in water,

i) from 0.05 to 4.0 mg/mL of Altenaria allergen,
ii) from 0.05 to 50 mg/mL of Aspergillus allergen,
iii) from 0.8 to 81.6 mg/mL of Bermuda grass allergen,
iv) from 0.1 to 6.0 mg/mL of birch allergen,
v) from 0.6 to 20.6 mg/mL of cat allergen,
vi) from 0.04 to 4.5 mg/mL of mountain cedar allergen,
vii) from 0.1 to 20.5 mg/mL of Japanese cedar allergen,
viii) from 0.05 to 38.4 mg/mL of Cladosporium allergen,
ix) from 1.3 to 38.4 mg/mL of dog allergen,
x) from 0.7 to 22.4 mg/mL of D. farinase allergen,
xi) from 0.6 to 84.2 mg/mL of D. pteronyssinus allergen,
xii) from 0.1 to 146.0 mg/mL of elm allergen,
xiii) from 0.02 to 0.2 mg/mL of feather allergen,
xiv) from 0.2 to 148.2 mg/mL of giant ragweed allergen,
xv) from 0.4 to 100 mg/mL of house dust allergen,
xvi) from 0.05 to 21.8 mg/mL of June/Kentucky bluegrass allergen,
xvii) from 0.2 to 47.0 mg/mL of lamb's quarters allergen,
xviii) from 0.1 to 166.3 mg/mL of maple allergen,
xix) from 0.3 to 90.4 mg/mL of mugwort allergen,
xx) from 0.1 to 12 mg/mL of mulberry allergen,
xxi) from 0.2 to 29.2 mg/mL of oak allergen,
xxii) from 0.1 to 66.8 mg/mL of olive allergen,
xxiii) from 1.0 to 40.0 mg/mL of Parietaria allergen,
xxiv) from 1.7 to 130.4 mg/mL of plantain allergen,
xxv) from 0.1 to 4.8 mg/mL of Penicillium allergen,
xxvi) from 0.05 to 17.3 mg/mL of perennial rye allergen,
xxvii) from 0.2 to 151.6 mg/mL of short ragweed allergen, or
xxviii) from 0.05 to 6.6 mg/mL of timothy allergen,.

19. An allergen composition, comprising:

a) a solvent; and
b) an allergen solubilized in said solvent, thereby forming an allergen solution; and
c) wherein said allergen solution is combined with a pretreatment substance to form an allergen composition; wherein said pretreatment substance is selected from the group consisting of: denaturants excluding organic solvents and concentrated salt solutions; organic solvents; crosslinking agents; concentrated salt solutions; and combinations thereof;

the allergen composition is used for the in vitro detection of the presence or amount of IgE in a test sample.

20. The allergen composition according to claim 19, wherein:

a) said allergen is selected from the group consisting of Altenaria allergen, birch allergen and dog allergen; and
b) said pretreatment substance is a formaldehyde solution.

21. The allergen composition according to claim 20, wherein said pretreatment substance is about 10 to about 20 microliters of a 37 percent formaldehyde solution.

22. The allergen composition according to claim 20, wherein said pretreatment substance is about 12.5 microliters of a 37 percent formaldehyde solution.

23. The allergen composition according to claim 19, wherein:

a) said allergen is selected from the group consisting of Bermuda grass allergen; Japanese cedar allergen; June/Kentucky bluegrass allergen; perennial rye allergen; and timothy allergen; and
b) said pretreatment substance is tetrahydrofuran.

24. The allergen composition according to claim 23, wherein said pretreatment substance is about 10 to about 50 microliters of tetrahydrofuran.

25. The allergen composition according to claim 23, wherein said pretreatment substance is about 25 microliters of tetrahydrofuran.

26. The allergen composition according to claim 19, wherein:

a) said allergen is selected from the group consisting of mountain cedar allergen; oak allergen and olive allergen; and
b) said pretreatment substance comprises tetrahydrofuran and a formaldehyde solution.

27. The allergen composition according to claim 26, wherein said pretreatment substance comprises about 10 to about 50 microliters of tetrahydrofuran and about 10 to about 20 microliters of a 37 percent formaldehyde solution.

28. The allergen composition according to claim 26, wherein said pretreatment substance comprises about 25 microliters of tetrahydrofuran and about 15 microliters of a 37 percent formaldehyde solution.

29. The allergen composition according to claim 19, wherein:

a) said allergen is selected from the group consisting of Cladosporium allergen and feather allergen; and
b) said pretreatment substance is about 0.5M to about 10M sodium chloride solution.

30. The allergen composition according to claim 29, wherein said pretreatment substance is about 10 to about 20 microliters of 5M sodium chloride.

31. The allergen composition according to claim 29, wherein said pretreatment substance is about 12 microliters of 5M sodium chloride.

32. The allergen composition according to claim 19, wherein:

a) said allergen is selected from the group consisting of *Dermatophagoides farinae* allergen and *D. pteronyssinus* allergen; and
b) said pretreatment substance comprises 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide and sodium borohydride.

33. The allergen composition according to claim 32, wherein said pretreatment substance comprises about 5.0 to about 15 microliters of 50 mg/mL 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide and about 1.0 to about 5.0 microliters of 20 mg/mL sodium borohydride.

34. The allergen composition according to claim 32, wherein said pretreatment substance comprises about 10 microliters of 50 mg/mL 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide and about 2.0 microliters of 20 mg/mL sodium borohydride.

35. The allergen composition according to claim 19, wherein:

a) said allergen is selected from the group consisting of lamb's quarters allergen and mulberry allergen; and

b) said pretreatment substance comprises acetic acid, buffered to about a pH of 7.

36. The allergen composition according to claim **35**, wherein said pretreatment substance comprises about 5.0 to about 30 microliters of acetic acid and 6N aqueous sodium hydroxide at about a pH of 7.

37. The allergen composition according to claim **35**, wherein said pretreatment substance comprises about 12.5 microliters of acetic acid and 6N aqueous sodium hydroxide to a pH of 7.

38. The allergen composition according to claim **19**, wherein:

a) said allergen is selected from the group consisting of Parietaria allergen and Penicillium allergen; and

b) said pretreatment substance comprises hydrochloric acid, buffered to about a pH of 7.

39. The allergen composition according to claim **38**, wherein said pretreatment substance comprises about 6.0 to about 30 microliters of 6N hydrochloric acid and 6N aqueous sodium hydroxide at about a pH of 7.

40. The allergen composition according to claim **38**, wherein said pretreatment substance comprises about 24 microliters of 6N hydrochloric acid and 6N aqueous NaOH at about a pH of 7.

41. An allergen composition, comprising:

a) a solvent;

b) an allergen solubilized in said solvent, thereby forming an allergen solution; and

c) a pretreatment substance selected from the group consisting of: denaturants excluding organic solvents and concentrated salt solutions; organic solvents; crosslinking agents; concentrated salt solutions; and combinations thereof;

wherein said allergen solution is combined with said pretreatment substance to form an allergen composition, and wherein the allergen composition is used for the in vitro detection of the presence or amount of IgE in a test sample.

42. A method for determining the presence or amount of IgE in a test sample, comprising the steps of:

a) providing at least one allergen immobilized upon a solid phase comprising a material selected from the group consisting of nitrocellulose, nitrocellulose derivatives, nitrocellulose compounds, and combinations thereof, wherein said allergen is applied to said solid phase as an allergen composition; said allergen composition being formed from combining an allergen with a pretreatment substance; wherein said pretreatment substance is selected from the group consisting of: denaturants excluding organic solvents and concentrated salt solutions; organic solvents; cross-linking agents, concentrated salt solutions; and combinations thereof containing, on the basis of allergen protein in a solvent,

i) from 0.05 to 4.0 mg/mL of Altenaria allergen,

ii) from 0.05 to 50 mg/mL of Aspergillus allergen,

iii) from 0.8 to 81.6 mg/mL of Bermuda grass allergen,

iv) from 0.1 to 6.0 mg/mL of birch allergen,

v) from 0.6 to 20.6 mg/ml of cat allergen,

vi) from 0.04 to 4.5 mg/ml of mountain cedar allergen,

vii) from 0.1 to 20.5 mg/ml of Japanese cedar allergen,

viii) from 0.05 to 38.4 mg/ml of Cladosporium allergen,

ix) from 1.3 to 38.4 mg/ml of dog allergen,

x) from 0.7 to 22.4 mg/ml of D. farinase allergen,

xi) from 0.6 to 84.2 mg/ml of D. pteronyssinus allergen,

xii) from 0.1 to 146.0 mg/ml of elm allergen,

xiii) from 0.02 to 0.2 mg/ml of feather allergen,

xiv) from 0.2 to 148.2 mg/ml of giant ragweed allergen,

xv) from 0.4 to 100 mg/ml of house dust allergen,

xvi) from 0.05 to 21.8 mg/ml of June/Kentucky bluegrass allergen,

xvii) from 0.2 to 47.0 mg/ml of lamb's quarters allergen,

xviii) from 0.1 to 166.3 mg/ml of maple allergen,

xix) from 0.3 to 90.4 mg/ml of mugwort allergen,

xx) from 0.1 to 12 mg/ml of mulberry allergen,

xxi) from 0.2 to 29.2 mg/ml of oak allergen,

xxii) from 0.1 to 66.8 mg/ml of olive allergen,

xxiii) from 1.0 to 40.0 mg/ml of Parietaria allergen,

xxiv) from 1.7 to 130.4 mg/ml of plantain allergen,

xxv) from 0.1 to 4.8 mg/ml of Penicillium allergen,

xxvi) from 0.05 to 17.3 mg/ml of perennial rye allergen,

xxvii) from 0.2 to 151.6 mg/ml of short ragweed allergen, or

xxviii) from 0.05 to 6.6 mg/ml of timothy allergen, and

b) contacting said test sample to said solid phase, thereby immobilizing allergen-specific IgE antibody from the test sample upon said solid phase by forming allergen/antibody complexes; and

c) detecting said immobilized allergen-specific antibody to determine the presence or amount of the antibody in the test sample.

43. The method according to claim **42**, wherein step c) comprises contacting said solid phase with an indicator reagent to determine the presence or amount of IgE in the test sample.

44. The method according to claim **43**, wherein said indicator reagent comprises a label conjugated to a binding member specific for a member selected from the group consisting of allergen, IgE and an ancillary specific binding member.

45. The method according to claim **44**, wherein said indicator reagent comprises a label conjugated to an anti-IgE antibody or anti-IgE antibody fragment.

46. The method according to claim **45**, wherein free or bound labeled anti-IgE antibody is detected to determine the presence or amount of IgE in the test sample.

47. The method according to claim **44**, wherein said label is a member selected from the group consisting of chromogens, catalysts, fluorescent compounds, chemiluminescent compounds, radioactive isotopes, colloidal metallic particles, colloidal selenium particles, dye particles, enzymes, substrates, organic polymer latex particles and liposomes or other vesicles containing signal producing components.

48. The method according to claim **43**, further comprising the step of washing unbound indicator reagent from said solid phase prior to detecting the presence or amount of IgE in the test sample.

49. The method according to claim **42**, wherein said solid phase is a member selected from the group consisting of cellulose, cellulose derivatives, silica, fiberglass, a porous polymer matrix, porous gels, polymeric films, agarose and porous fibrous matrixes.

50. A method for determining the presence or amount of IgE in a test sample, comprising the steps of:

a) providing at least one allergen immobilized upon a solid phase, wherein said solid phase comprises a material selected from the group consisting of nitrocellulose, nitrocellulose derivatives, nitrocellulose compounds, and combinations thereof, wherein said allergen is applied to said solid phase as an allergen composition comprising.:

i) a solvent;

ii) an allergen solubilized in said solvent, thereby forming an allergen solution; and

iii) a pretreatment substance selected from the group consisting of: denaturants excluding organic solvents and concentrated salt solutions; organic solvents, crosslinking agents; concentrated salt solutions, and combinations thereof, wherein said allergen solution is combined with said pretreatment substance to form an allergen composition;

b) contacting the test sample to said solid phase, thereby immobilizing allergen-specific IgE antibody from the test sample upon said solid phase by forming allergen/antibody complexes; and

c) detecting said immobilized allergen-specific antibody to determine the presence or amount of the antibody in the test sample.

51. A kit for determining the presence or amount of IgE in a test sample, comprising:

a) an allergen immobilized upon a solid phase comprising a material selected from the group consisting of nitrocellulose, nitrocellulose derivatives, nitrocellulose compounds, and combinations thereof, wherein said allergen is applied to said solid phase as an allergen composition comprising:

i) a solvent;

ii) an allergen solubilized in said solvent, thereby forming an allergen solution; and

iii) a pretreatment substance selected from the group consisting of: denaturants excluding organic solvents and concentrated salt solutions; organic solvents; crosslinking agents; concentrated salt solution,; and combinations thereof, wherein said allergen solution is combined with said pretreatment substance to form an allergen composition; and

b) an indicator reagent in a container, wherein said indicator reagent is used to determine the presence or amount of IgE in the test sample, wherein said indicator reagent comprises a label conjugated to a binding member specific for a member selected from the group consisting of the allergen, IgE and an ancillary specific binding member.

* * * * *

US005445970A

United States Patent [19]

Rohr

[11] **Patent Number: 5,445,970**

[45] **Date of Patent: Aug. 29, 1995**

[54] **MAGNETICALLY ASSISTED BINDING ASSAYS USING MAGNETICALLY LABELED BINDING MEMBERS**

[75] Inventor: **Thomas E. Rohr**, Ferndale, Ill.

[73] Assignee: **Abbott Laboratories**, Abbott Park, Ill.

[21] Appl. No.: **348,503**

[22] Filed: **Dec. 1, 1994**

Related U.S. Application Data

[63] Continuation of Ser. No. 161,105, Dec. 2, 1993, abandoned, which is a continuation-in-part of Ser. No. 854,151, Mar. 20, 1992, abandoned.

[51] **Int. Cl.**[6] **G01N 33/546**; G01N 33/553

[52] **U.S. Cl.** **436/526**; 436/528; 436/534; 436/806; 422/236; 209/214

[58] **Field of Search** 436/526, 528, 534, 806; 422/236; 209/214; 210/222, 223, 695

[56] **References Cited**

U.S. PATENT DOCUMENTS

5,236,824 8/1993 Fujiwara et al. 435/5
5,252,493 10/1993 Fujiwara et al. 436/526

Primary Examiner—Toni R. Scheiner
Assistant Examiner—Susan C. Wolski
Attorney, Agent, or Firm—Mark C. Bach

[57] **ABSTRACT**

The present invention provides assay methods for performing binding assays, wherein the detectable label is a magnetically responsive material. Direct and indirect, competitive and sandwich assay formats are used to partition the magnetically attractable label between a solid phase and a fluid phase in proportion to the presence or amount of analyte in the test sample. The magnetic responsiveness of the magnetically attractable label in one or both phases results in the exertion of a force upon the label. By determining the extent of the force or influence of the force exerted upon the label, the amount of the analyte in the test sample is determined.

8 Claims, 12 Drawing Sheets

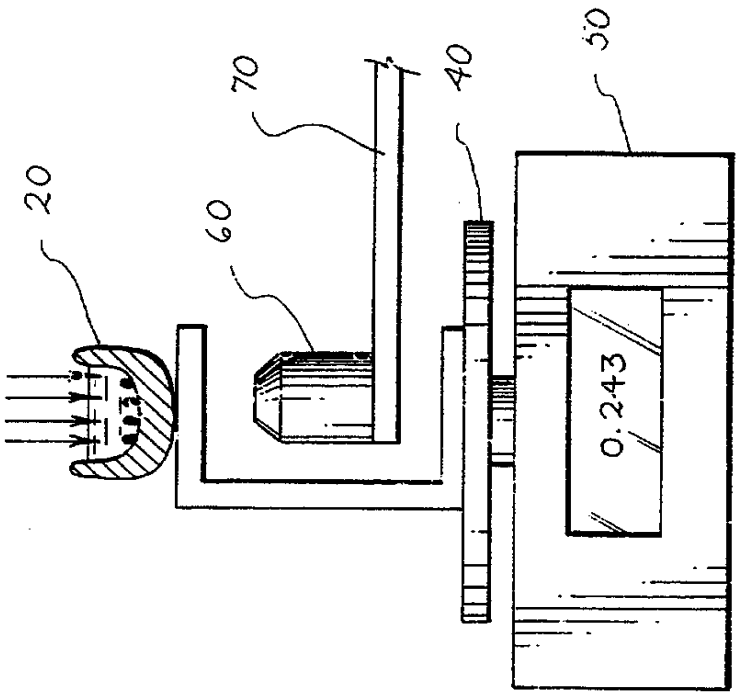


Fig. 2

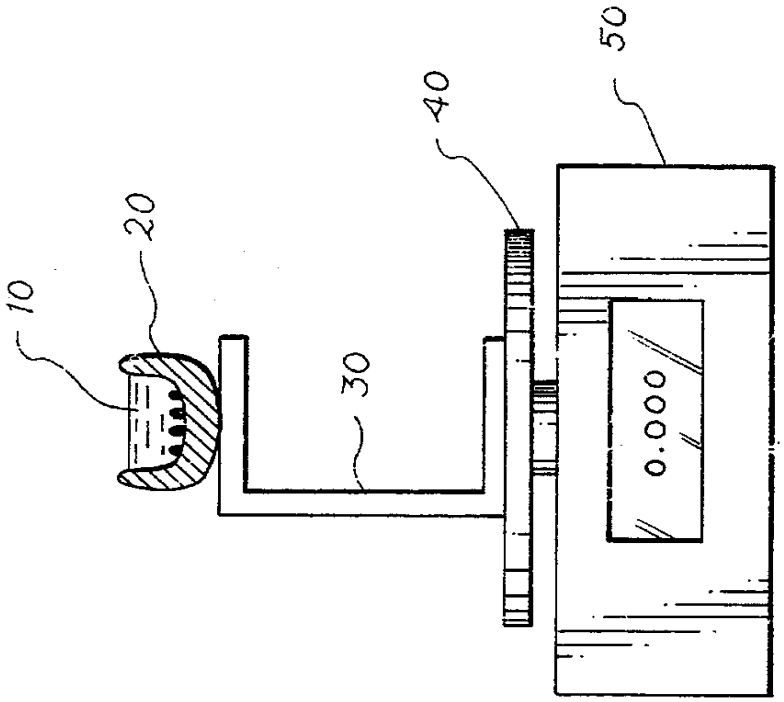
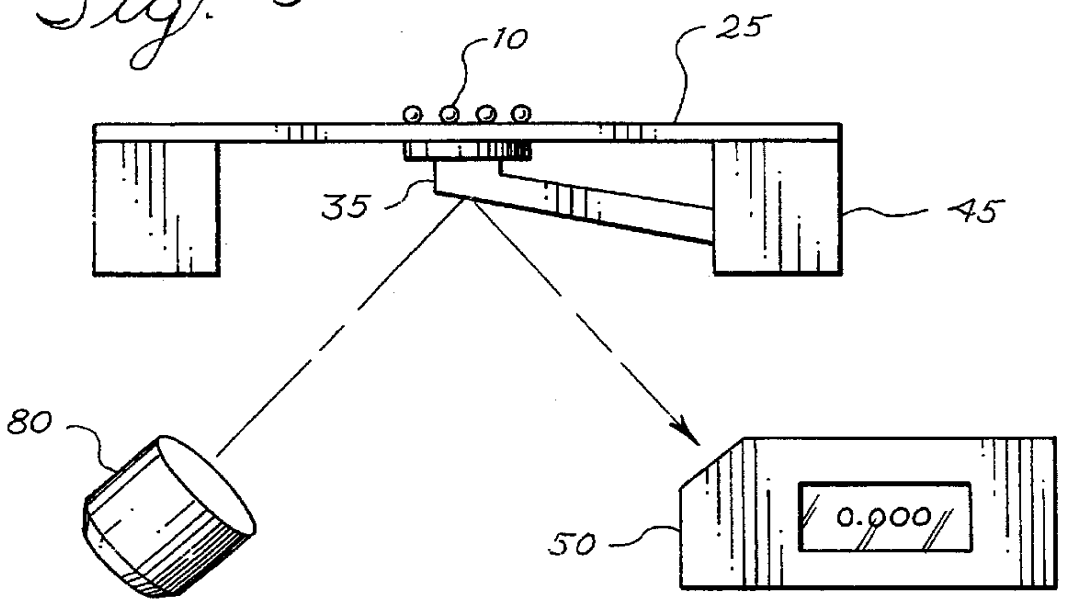


Fig. 1

Fig. 3



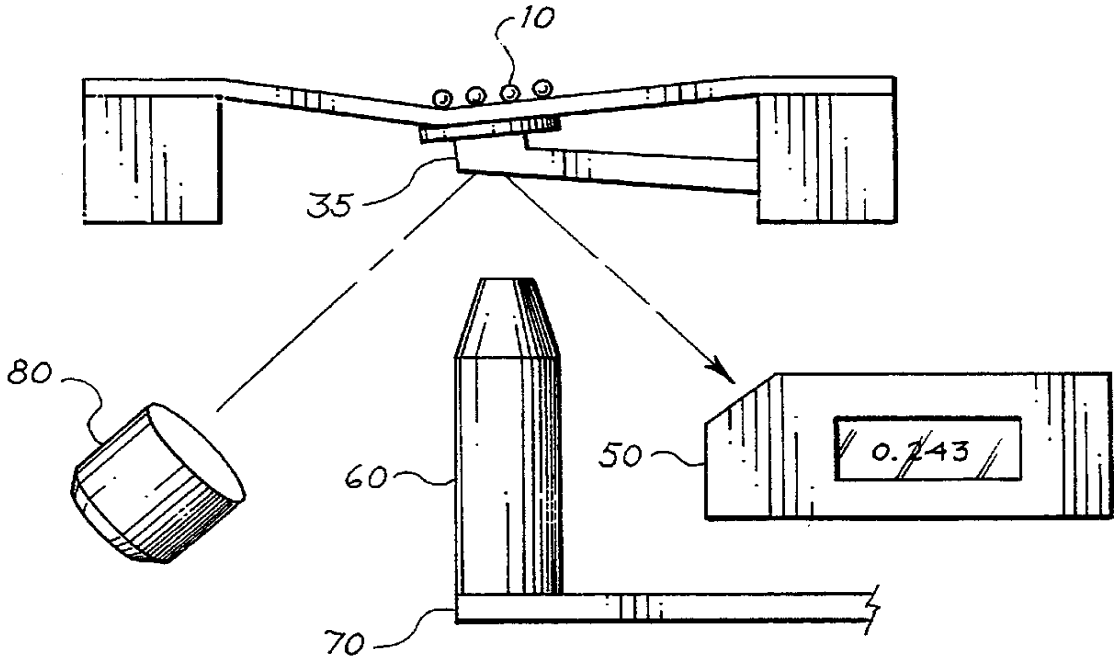


Fig. 4

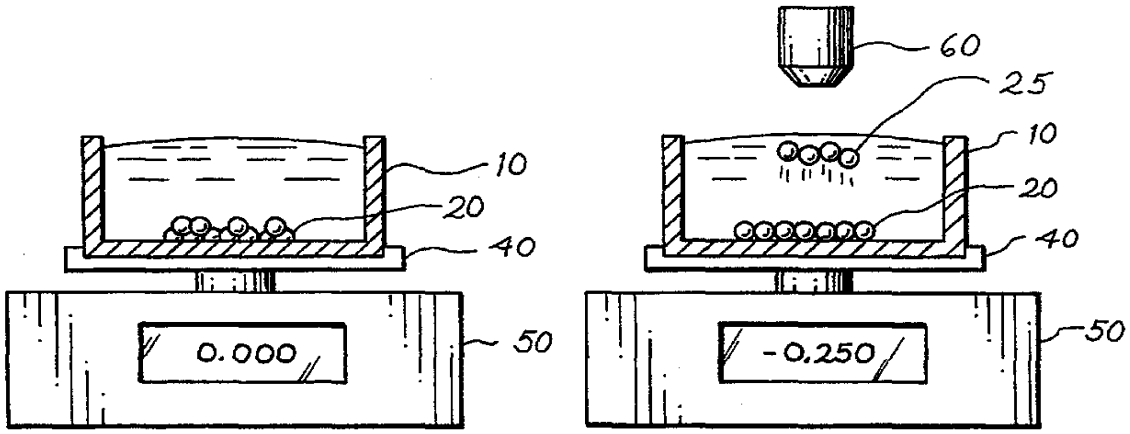


Fig. 5a

Fig. 5b

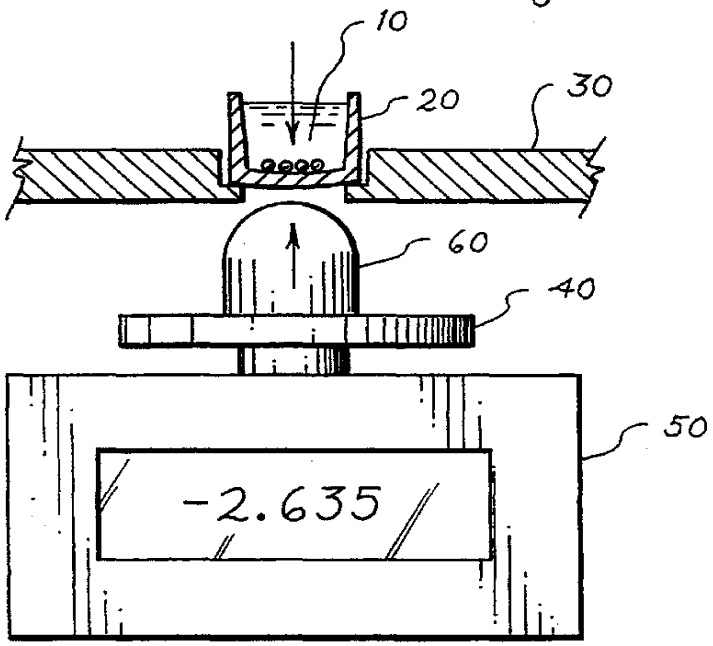


Fig. 16

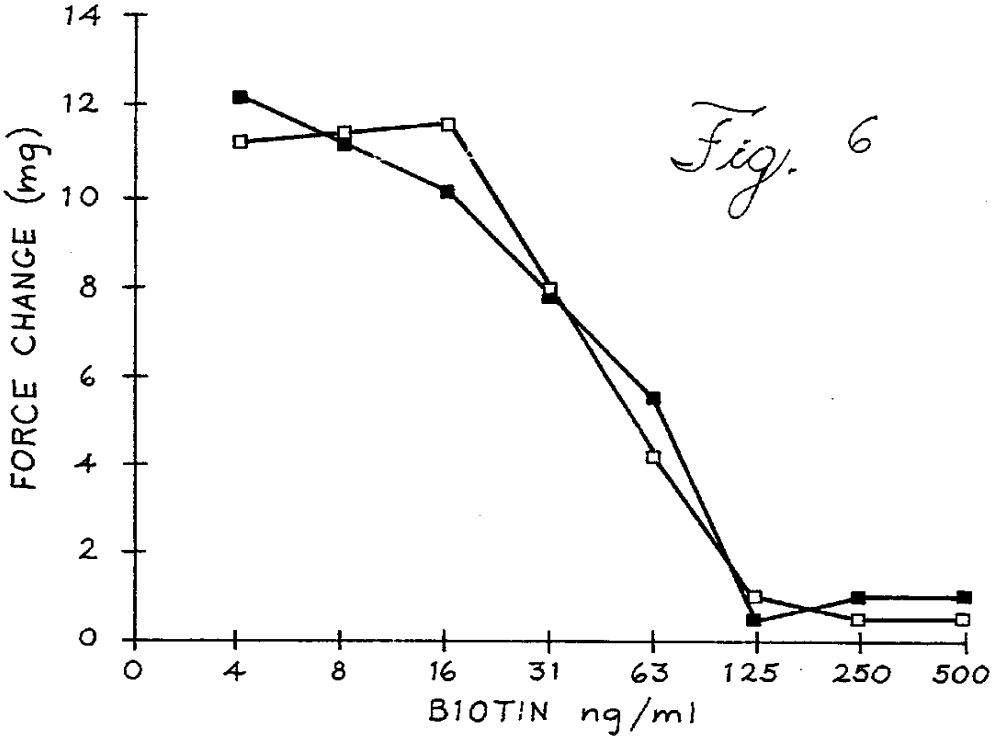

FORCE CHANGE (mg)
14
12
10
8
6
4
2
0
0
4
8
16
31
63
125
250
500
BIOTIN ng/ml

Fig. 6

Fig. 7

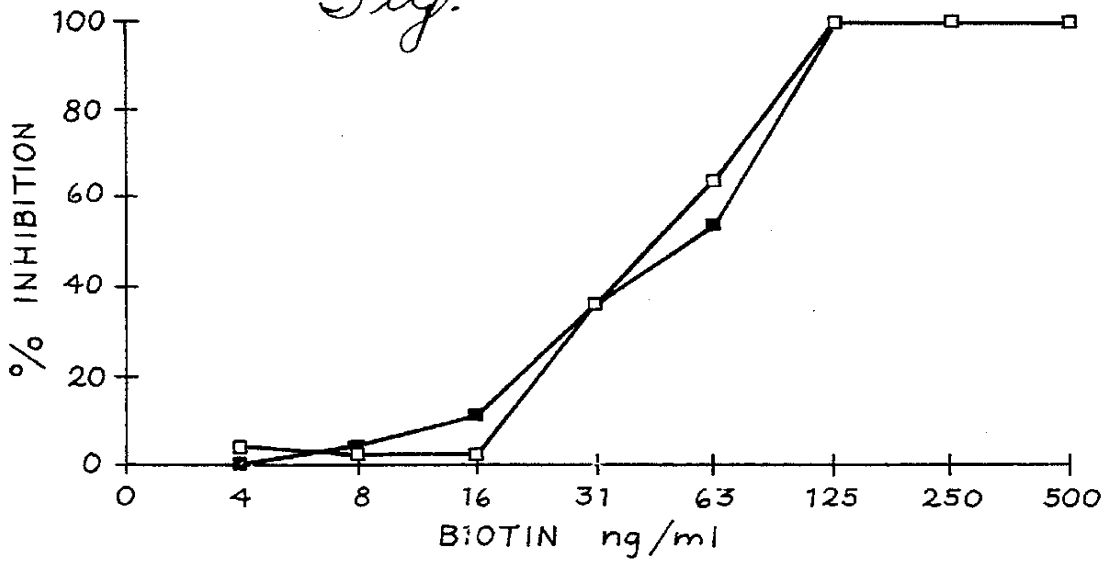

% INHIBITION
100
80
60
40
20
0
0
4
8
16
31
63
125
250
500
BIOTIN ng/ml

Fig. 8

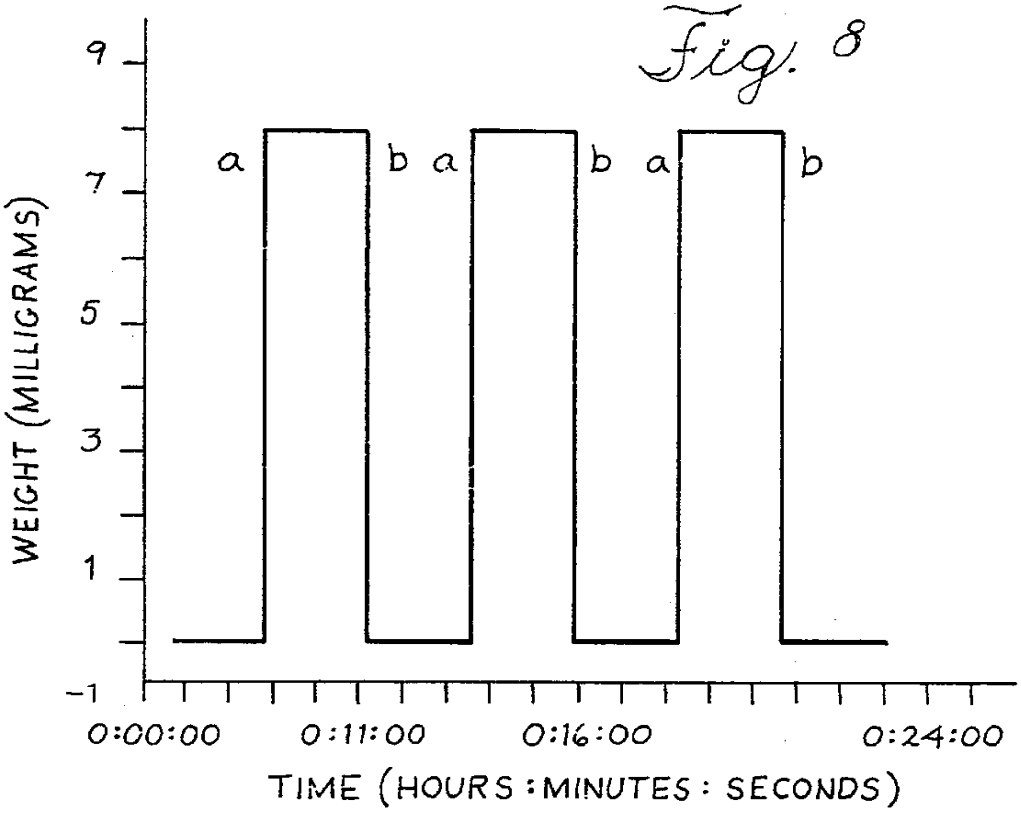

9
7
5
3
1
-1
WEIGHT (MILLIGRAMS)
a
b a
b a
b
0:00:00
0:11:00
0:16:00
0:24:00
TIME (HOURS : MINUTES : SECONDS)

Fig. 9

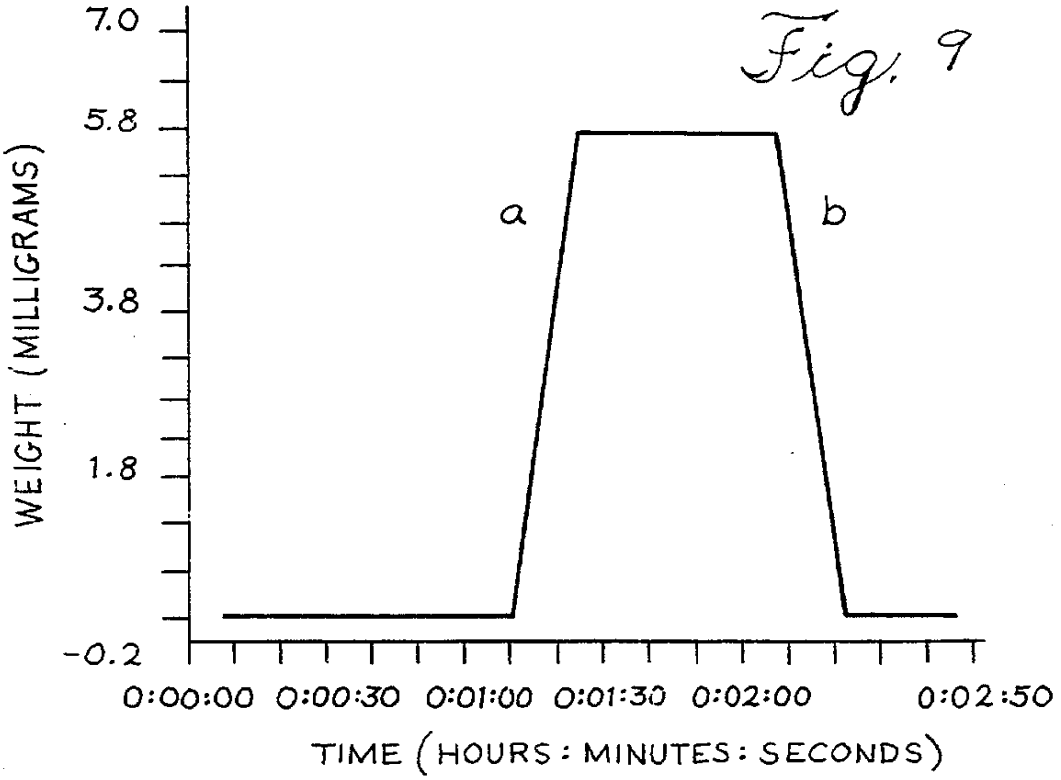

7.0
5.8
3.8
1.8
-0.2
WEIGHT (MILLIGRAMS)
a
b
0:00:00
0:00:30
0:01:00
0:01:30
0:02:00
0:02:50
TIME (HOURS : MINUTES : SECONDS)

Fig. 10

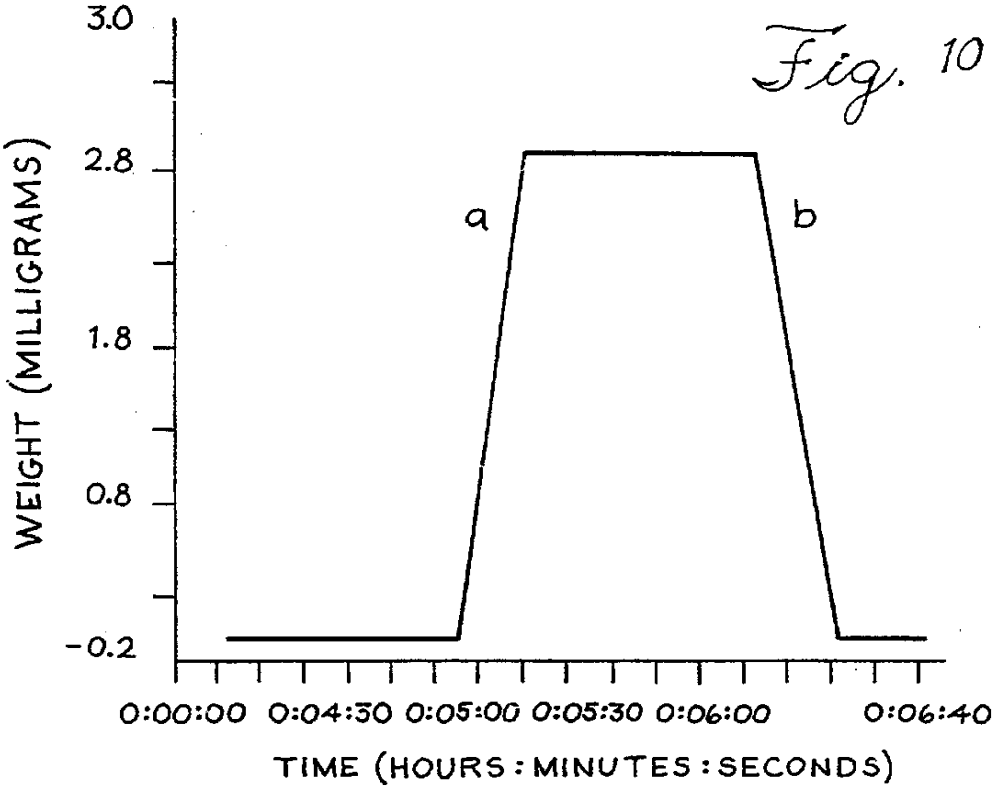
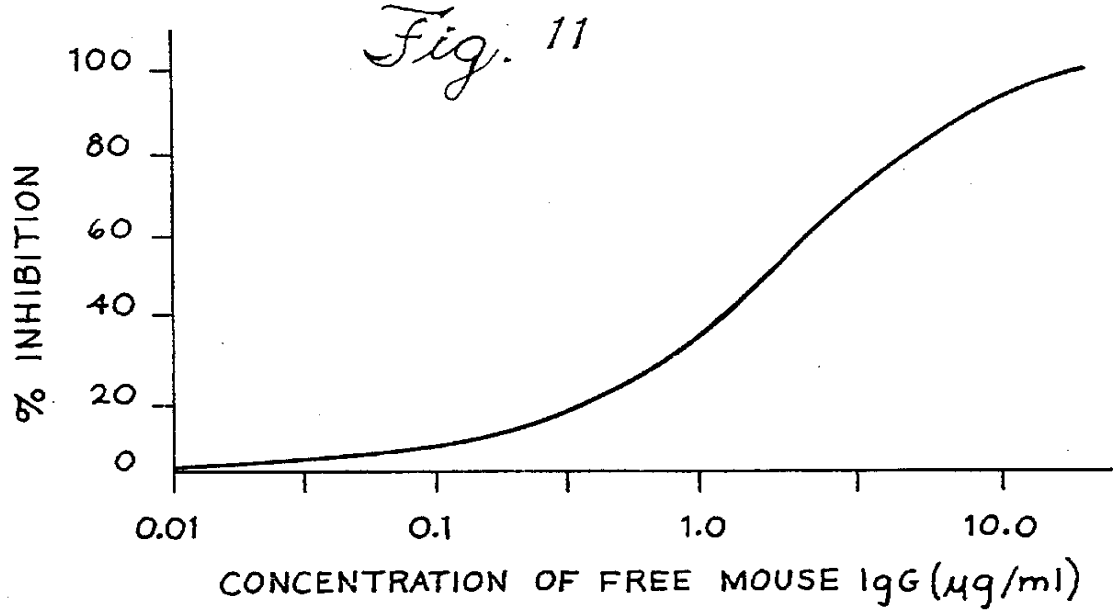

3.0
2.8
1.8
0.8
-0.2
WEIGHT (MILLIGRAMS)
a
b
0:00:00 0:04:30 0:05:00 0:05:30 0:06:00 0:06:40
TIME (HOURS : MINUTES : SECONDS)

Fig. 11


100
80
60
40
20
0
% INHIBITION
0.01
0.1
1.0
10.0
CONCENTRATION OF FREE MOUSE IgG (μg/ml)

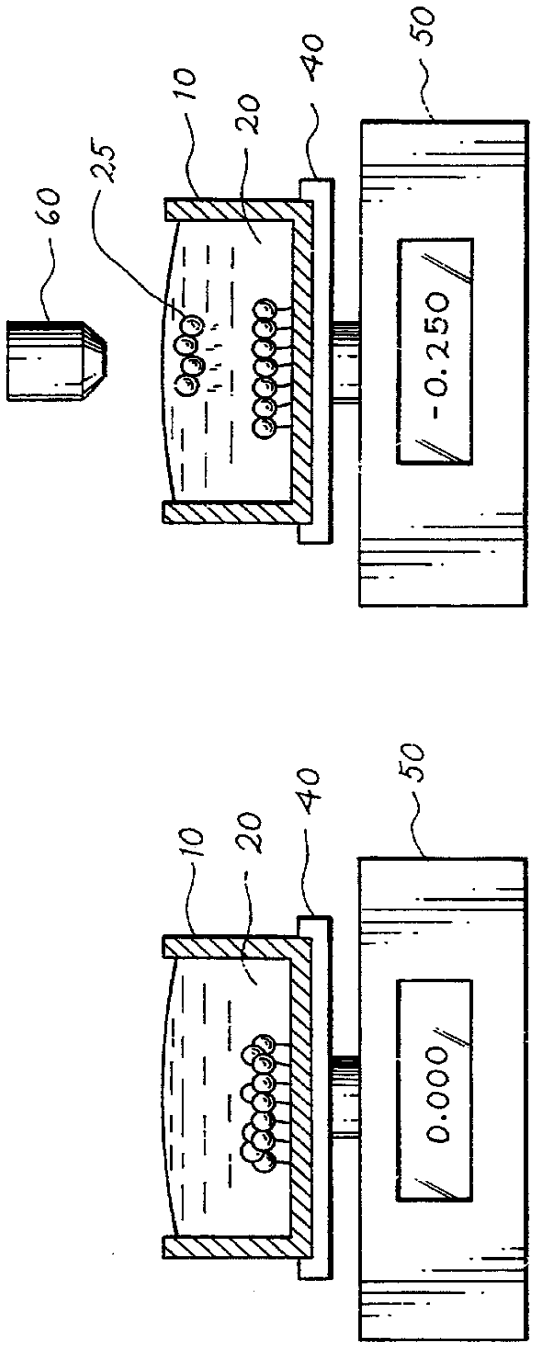


Fig. 12b

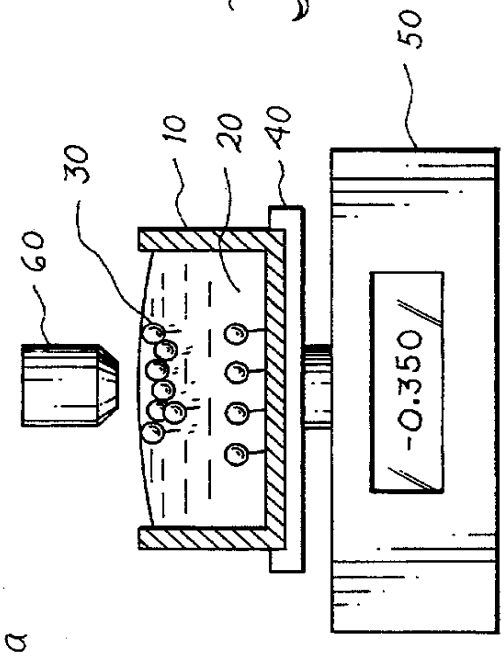


Fig. 12c

Fig. 12a

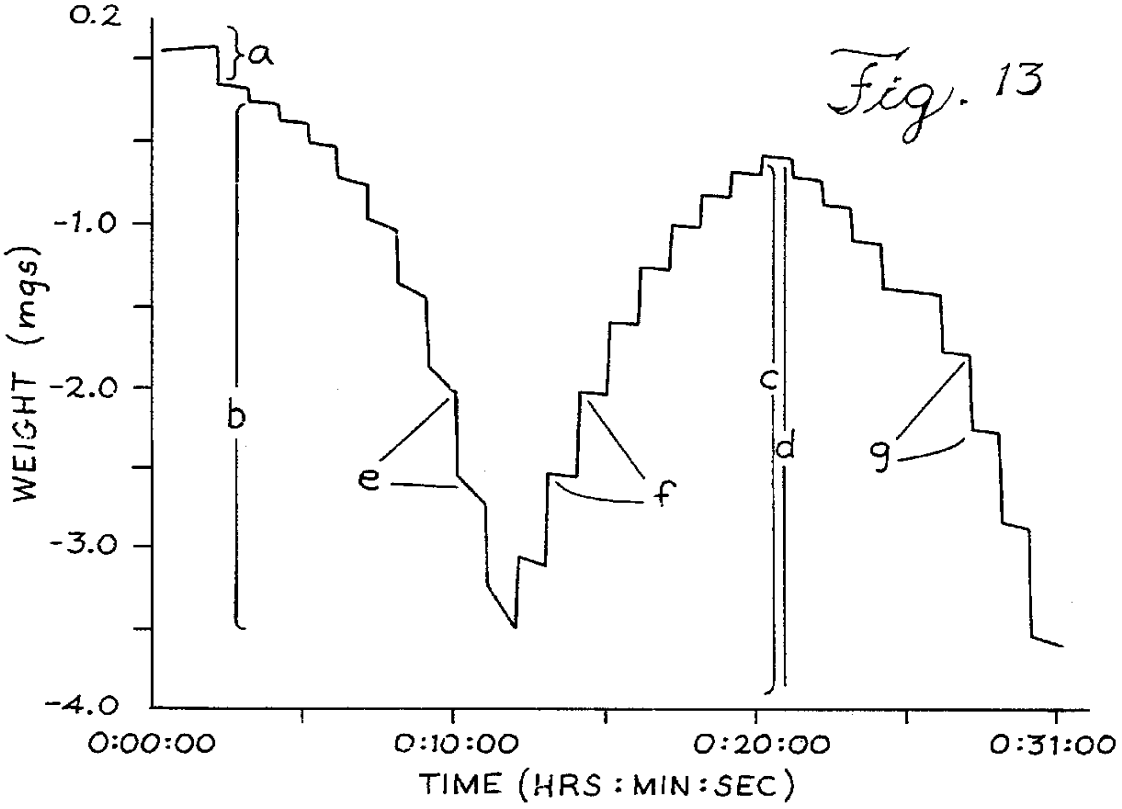

0.2
-1.0
-2.0
-3.0
-4.0
WEIGHT (mgs)
a
b
c
d
e
f
g
0:00:00
0:10:00
0:20:00
0:31:00
TIME (HRS : MIN : SEC)

Fig. 13

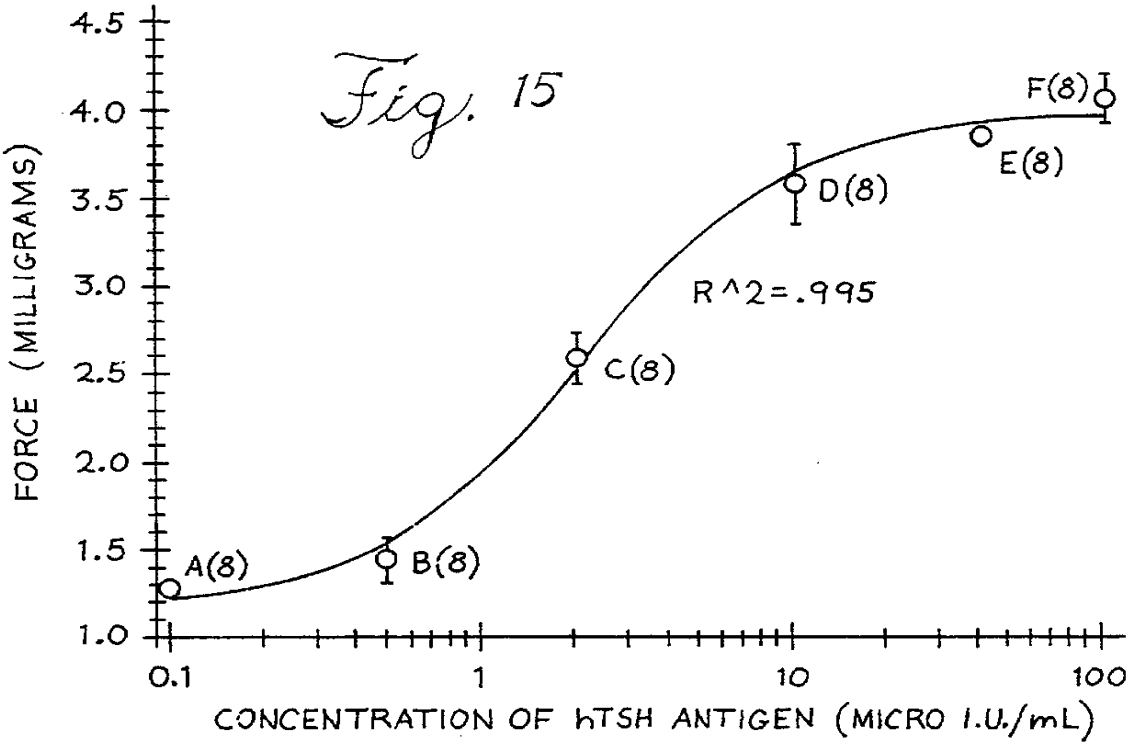
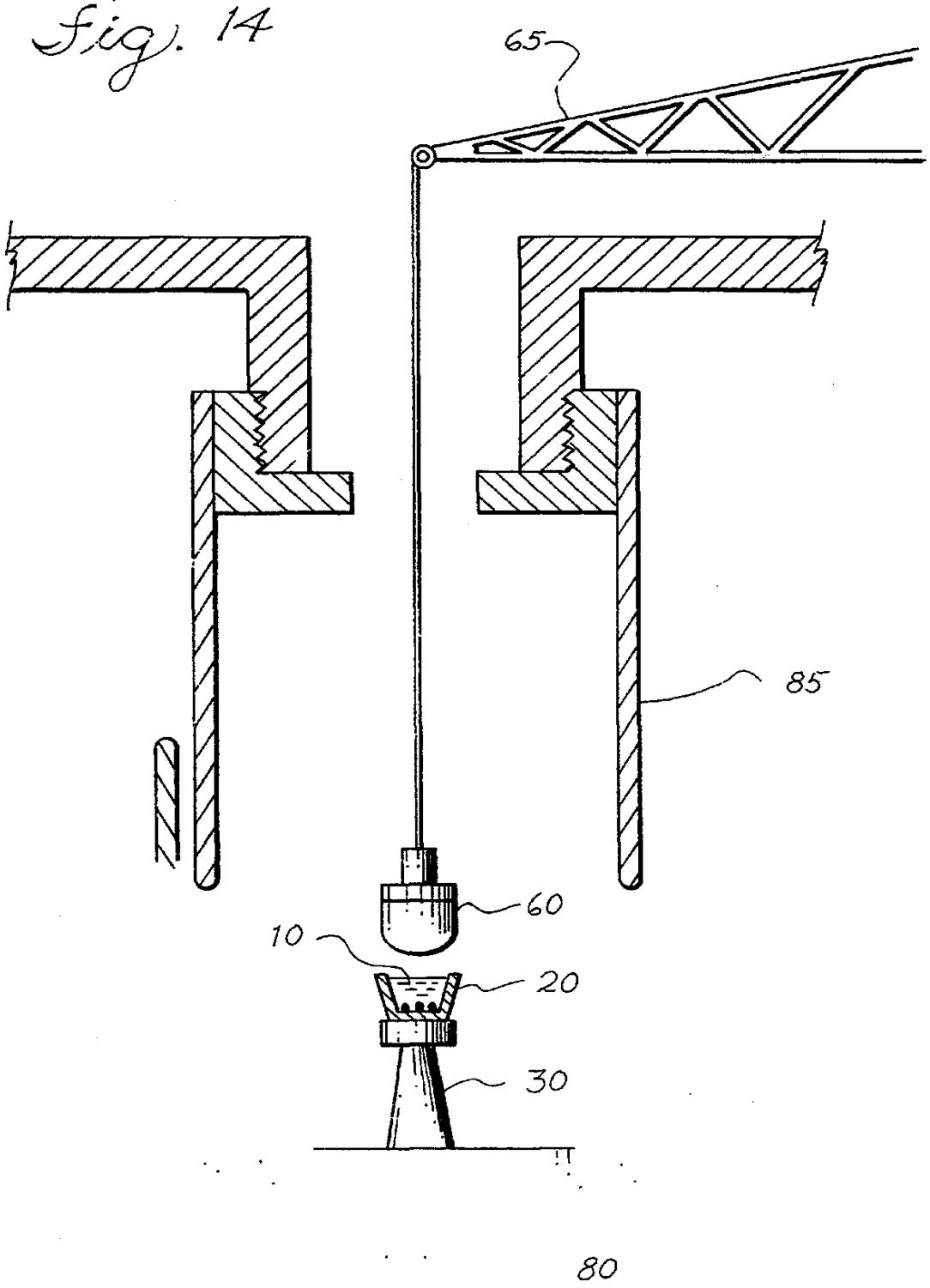

4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
FORCE (MILLIGRAMS)
A(8)
B(8)
C(8)
D(8)
E(8)
F(8)
R^2=.995
0.1
1
10
100
CONCENTRATION OF hTSH ANTIGEN (MICRO I.U./mL)

Fig. 15

Fig. 14



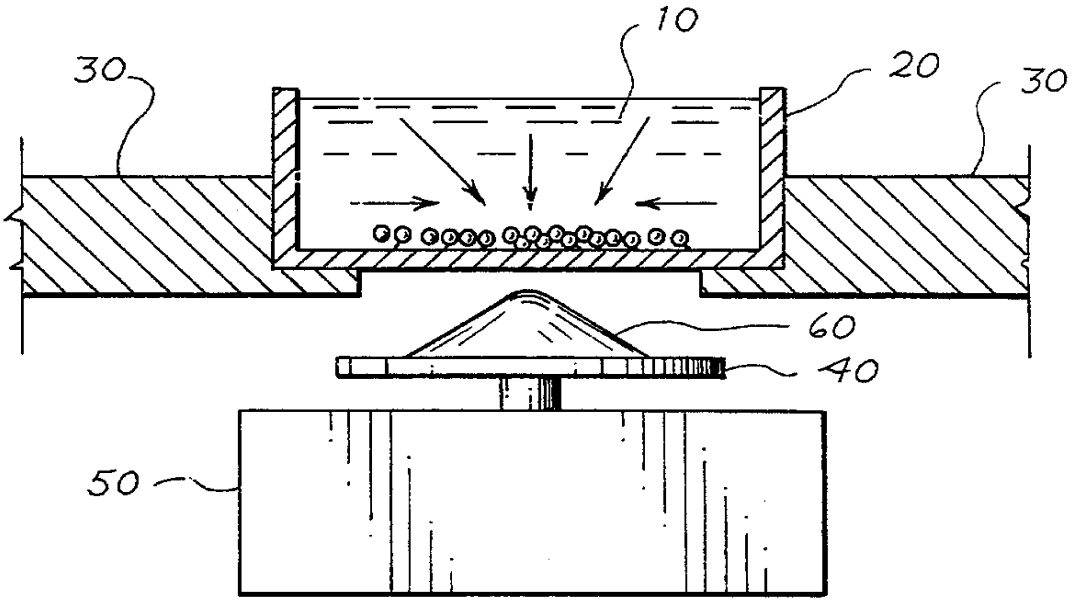


Fig. 17a

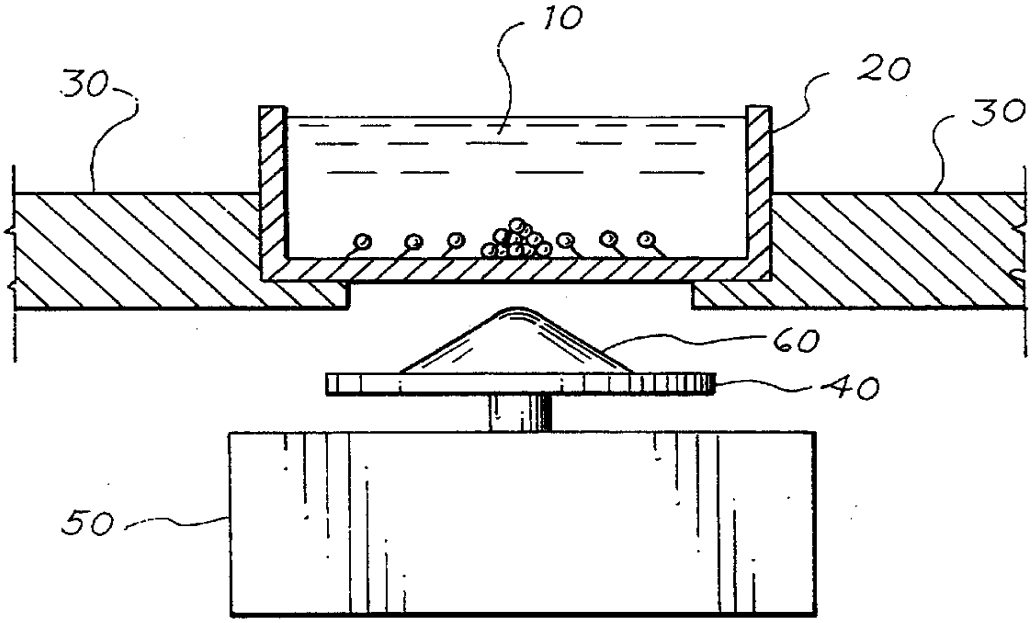


Fig. 17b

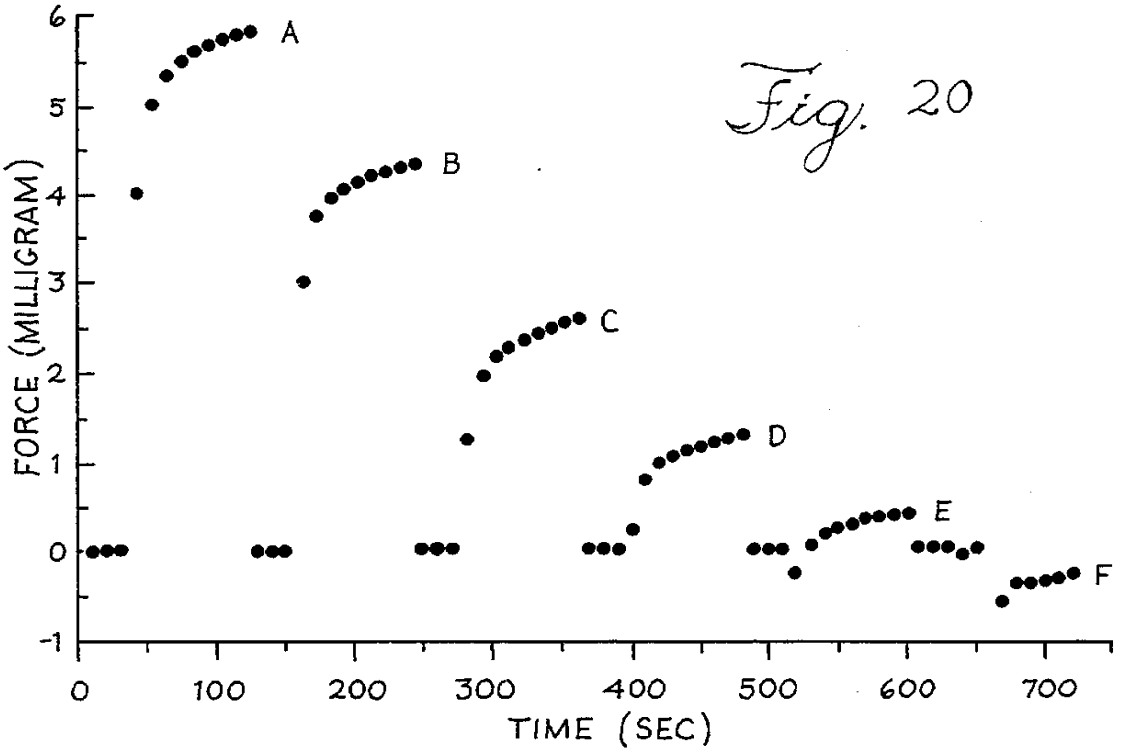

FORCE (MILLIGRAM)
TIME (SEC)
A
B
C
D
E
F

Fig. 20

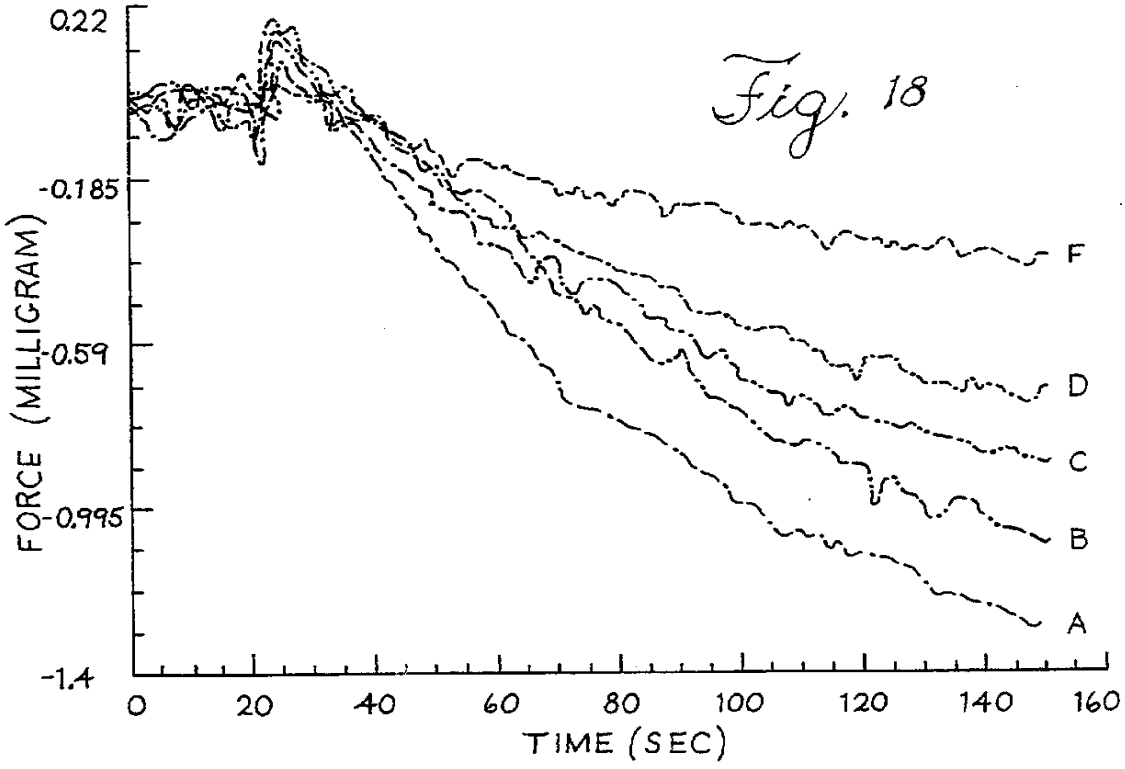

FORCE (MILLIGRAM)
TIME (SEC)
F
D
C
B
A

Fig. 18

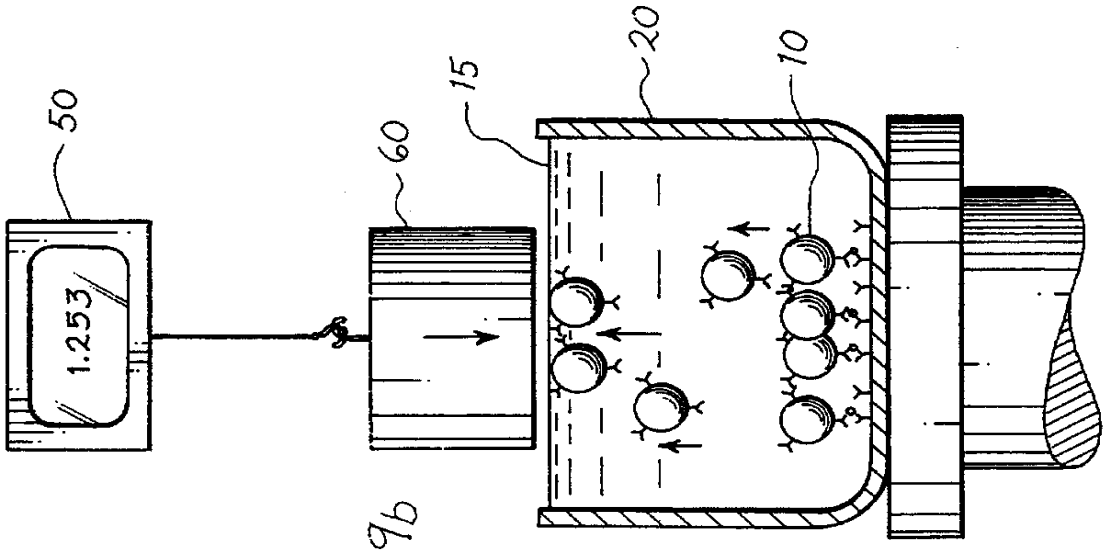


Fig. 19b

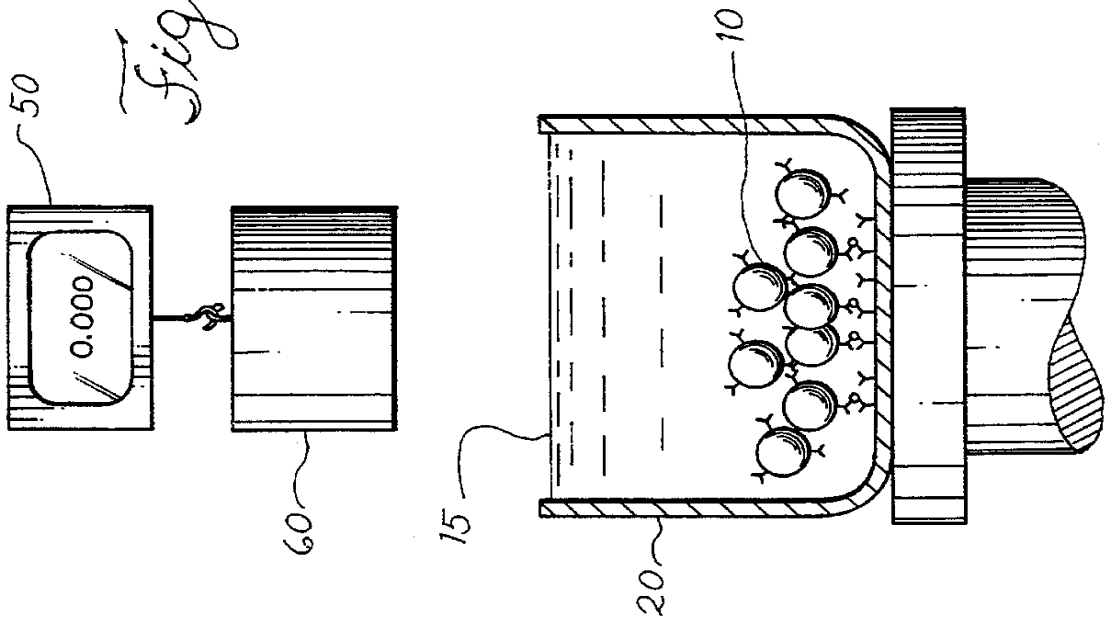


Fig. 19a

MAGNETICALLY ASSISTED BINDING ASSAYS USING MAGNETICALLY LABELED BINDING MEMBERS

This application is a continuation of application Ser. No.: 08/161,105, filed on Dec. 2, 1993, now abandoned, which is a continuation-in-part of U.S. patent application Ser. No. 07/854,151, filed Mar. 20, 1992, now abandoned.

FIELD OF THE INVENTION

This invention relates to a method for determining the presence or amount of analyte in a test sample using a detectable label attached to a binding member. In particular, the invention relates to the use of magnetically-attractable materials as the detectable label.

BACKGROUND OF THE INVENTION

Diagnostic assays have become an indispensable means for detecting analytes in test samples by using the mutual reaction between the analyte and a specific binding member for the analyte, such as the immunoreaction between an antigen and the antibody to that antigen. Typically, detectable tags or labels attached to antibodies, which in turn bind to the analyte of interest, are employed in such diagnostic assays, wherein the detection of the resultant labeled antibody/analyte complex, or of the labeled antibody which remains unbound, is used to indicate the presence or amount of analyte in the test sample.

Two commonly used binding assay techniques are the radioimmunoassay (RIA) and the enzyme immunoassay (EIA), both of which employ a labeled binding member. The RIA uses a radioactive isotope as the detectable tag or label attached to a binding member. Because the radioactive isotope can be detected in very small amounts, it can be used to detect or quantitate small amounts of analyte. However, substantial disadvantages associated with the RIA include the special facilities and extreme caution that are required in handling radioactive materials, the high costs of such reagents, and their unique disposal requirements.

The EIA uses an enzyme as the detectable tag or label attached to a binding member, wherein the enzymatic activity of the enzyme is used to detect the immunoreaction. While the EIA does not have the same disadvantages as the RIA, EIA techniques typically require the addition of substrate materials to elicit the detectable enzyme reaction. Enzyme substrates are also often unstable and have to be prepared just prior to use or be stored under refrigeration. In addition, enzyme labels may be difficult to purify and conjugate to binding members, and may be unstable during storage at room temperature. Enzyme immunoassays are also unsatisfactory in that the methods typically require complex incubations, multiple liquid additions and multiple wash steps. Moreover, even under refrigerated conditions, enzymes are unstable.

More recently, assay techniques using metallic sol particles as visual labels have been developed. In these techniques, a metal (e.g., gold, silver, platinum), a metal compound, or a nonmetallic substance coated with a metal or a metal compound, is used to form an aqueous dispersion of particles. Generally, the binding member to be labeled is coated onto the metal sol particles by adsorption, and the particles are captured or aggregated in the presence of analyte. Although the metal sol particles have the advantage of producing a signal that is visually detectable as well as measurable by an instrument, they, nevertheless, are difficult to quantitatively measure. The metallic particles also have a limited color intensity, and therefore limited sensitivity in some assays. In addition, the surfaces of inorganic metallic colloid particles, such as gold, do not readily accept the covalent attachment of binding members. Thus, during use in a binding assay, care must be taken so that the adsorbed binding members are not removed from the inorganic particles through the combination of displacement by other proteins or surface active agents and the shear forces which accompany washing steps used to remove non-specifically bound material. Sol particles can be difficult to coat without inducing aggregation, may aggregate upon storage and may aggregate upon the addition of buffers or salts. Furthermore, such particulate labels are difficult to concentrate, may aggregate during use and are difficult to disperse.

Other label materials include chemiluminescent and fluorescent substances. Non-metallic particles, such as dyed or colored latex and selenium particles have also been used as visual labels.

Prior to the present invention, magnetic particles and magnetic fields have generally been used as means to remove or position an analyte component of a test sample. For example, U.S. Pat. Nos. 4,070,246 and 3,985,649 describe the use of binding members attached to ferromagnetic particles, whereby the binding member forms a complex with the analyte of interest, and the resulting complex is removed from the reaction mixture by means of a magnetic field. Alternatively, U.S. Pat. No. 3,933,997 describes the use of magnetic particles and a magnetic field as a means of concentrating a radioactive material on a test substance. U.S. Pat. No. 4,219,335 describes the use of magnetic particles which have characteristics capable of affecting electrical resistance, wherein a capacitance measurement will reveal whether the particles are present on a surface. However, the effect of the magnetic field on the magnetic particles has no relation to the presence or amount of analyte in the test sample.

SUMMARY OF THE INVENTION

The present invention advantageously uses a magnetically-attractable material as a detectable label in binding assays. The magnetic label is subjected to a magnetic field and the label, in turn, displays a resultant force or movement as a result of the application of the magnetic field. According to the present invention, the extent of the force or movement is modulated by an analyte that may be present in a test sample. Because the presence or amount of analyte in a test sample is responsible for the magnitude of the force exerted or the amount of movement displayed by the attractable material, the effect of the magnetic field on the magnetically-attractable label can be used as a measure of the presence or amount of analyte in a test sample.

According to the present invention, a method of determining the presence or amount of analyte in a test sample comprises contacting a test sample with a solid-phase reagent and a magnetically-labeled reagent. The solid-phase reagent comprises a first binding member attached to a solid phase, and the magnetically-labeled reagent comprises a second binding member attached to a magnetically-attractable label. The first binding member is selected to bind (i) the analyte or (ii) the second binding member, and the second binding member is

selected to bind (i) the analyte or (ii) the first binding member, to thereby provide for competitive and sandwich immunoassay formats.

According to another method of the invention, determining the presence or amount of analyte in a test sample comprises contacting a test sample with a solid-phase reagent, a magnetically-labeled reagent and at least one ancillary binding member. The solid-phase reagent comprises a first binding member attached to a solid phase, and the magnetically-labeled reagent comprises a second binding member attached to a magnetically-attractable label. The first and second binding members are selected to bind either (i) at least one ancillary binding member or (ii) the analyte. The ancillary binding member is selected to bind (i).the analyte, (ii) the first binding member, or (iii) the second binding member, to thereby provide for competitive and sandwich immunoassay formats.

According to the method of the present invention, there is a partitioning of the magnetically-labeled reagent between unbound magnetically-labeled reagent and magnetically-labeled reagent that becomes bound or immobilized to the solid-phase reagent in relation to the amount of analyte present in the test sample. The unbound magnetically-labeled reagent can be separated from the magnetically-labeled reagent bound to the solid-phase reagent prior to or during the application of a magnetic field to the magnetically-labeled reagent. The magnetic field causes the magnetically-labeled reagent to respond in a manner that is related to the presence or amount of analyte present in the test sample. By detecting the extent of the magnetically-labeled reagent's response, the presence or amount of the analyte in a test sample can be determined. The extent of the magnetically-labeled reagent's response can be manifested in many measurable forms which can be detected using any means suitable for detecting the magnetically-labeled reagent's response to the magnetic field.

The present invention also provides devices for determining the presence or amount of an analyte in a test sample. Such devices comprise (i) a reaction vessel where unbound and immobilized magnetically-labeled reagent are produced in relation to the amount of analyte in the test sample; (ii) a separation means for partitioning the immobilized magnetically-labeled reagent and the unbound magnetically-labeled reagent; (iii) a magnetic field generator means for the application of a magnetic field to the magnetically-labeled reagent; and (iv) a measurement means to assess the effect of the magnetic field on the magnetically-labeled reagent as a measure of the presence or amount of the analyte in the test sample. It will be understood, of course, that the magnetic field generator means can also serve as the separation means and that suitable magnetic field generator means comprise permanent magnets and electromagnets.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** is a schematic view of the magnetically assisted detection of magnetically-labeled reagent using a balance or weighing means.

FIG. **2** is a schematic view of a balance means in operation for the magnetically assisted detection of a magnetically-labeled reagent.

FIG. **3** is a schematic view of the magnetically assisted detection of a magnetically-labeled reagent using an optical sensor means.

FIG. **4** is a schematic view of an optical sensor means in operation for the magnetically assisted detection of a magnetically-labeled reagent.

FIGS. **5***a* and **5***b* illustrate the measurement of the attractive force of unbound or free magnetically-labeled reagent.

FIG. **6** illustrates the results of a binding assay using a magnetically-labeled reagent as the detectable label.

FIG. **7** illustrates the results of a binding assay using a magnetically-labeled reagent as the detectable label, plotted as an inhibition curve.

FIG. **8** illustrates the effect of the repeated approach and withdrawal of a magnetic field from a solid phase containing antibody-coated magnetic particles captured by an immobilized antibody.

FIG. **9** illustrates a single cycle of the approach and withdrawal of a magnetic field from a solid phase containing antibody-coated magnetic particles captured by an immobilized antibody.

FIG. **10** illustrates the decrease in weight change due to the presence of free antibody during incubation in a system as illustrated in FIG. **9**.

FIG. **11** illustrates an inhibition curve from a magnetically assisted immunoassay.

FIGS. **12***a*, **12***b* and **12***c* illustrate a schematic view of the measurement means for the determination of the strength of associations between complementary binding members.

FIG. **13** illustrates an instrument tracing of the weight changes resulting from the approach of a magnet to the top of a vessel containing unbound and immobilized magnetically-labeled reagent.

FIG. **14** is a schematic view of the magnetically assisted detection of magnetically-labeled reagent using a field generator means suspended from a balance means.

FIG. **15** illustrates the results of a binding assay which was run using an apparatus comprising a field generator means suspended from a balance means.

FIG. **16** is a schematic view of the magnetically assisted detection of magnetically-labeled reagent using a field generator means positioned on a balance means.

FIGS. **17***a* and **17***b* are are schematic views of the magnetically assisted detection of magnetically-labeled reagent using a conical field generator means positioned on a balance means.

FIG. **18** illustrates the results of a binding assay which was run using an apparatus comprising a conical field generator means positioned on a balance means.

FIGS. **19***a–b* illustrate Example 11 discussed herein.

FIG. **20** illustrates rate of apparent weight change during performance of Example 11.

DETAILED DESCRIPTION OF THE INVENTION

The following definitions are applicable to the invention:

Definitions

The term "test sample", as used herein, refers to a material suspected of containing the analyte. The test sample can be used directly as obtained from the source or following a pre-treatment to modify the character of the sample. The test sample can be derived from any biological source, such as a physiological fluid including, but not intended to be limited to blood, saliva, ocular lens fluid, cerebral spinal fluid, sweat, urine, milk, ascites fluid, mucous, synovial fluid, peritoneal fluid, amniotic fluid and the like; fermentation broths;

cell cultures; chemical reaction mixtures and the like. The test sample can be pretreated prior to use, such as preparing plasma from blood, diluting viscous fluids, and the like. Methods of treatment can involve filtration, distillation, concentration, inactivation of interfering components, and the addition of reagents. In addition to biological or physiological fluids, other liquid samples can be used such as water, food products and the like for the performance of environmental or food production assays. In addition, a solid material suspected of containing the analyte can be used as the test sample. In some instances, it may be beneficial to modify a solid test sample to form a liquid medium or to release the analyte.

The term "binding member", as used herein, refers to a member of a binding pair, i.e., two different molecules wherein one of the molecules specifically binds to the second molecule through chemical or physical means. In addition to the well-known antigen and antibody binding pair members, other binding pairs include, but are not intended to be limited to, biotin and avidin, carbohydrates and lectins, complementary nucleotide sequences, complementary peptide sequences, effector and receptor molecules, enzyme cofactors and enzymes, enzyme inhibitors and enzymes, a peptide sequence and an antibody specific for the sequence or the entire protein, polymeric acids and bases, dyes and protein binders, peptides and specific protein binders (e.g., ribonuclease, S-peptide and ribonuclease S-protein), sugar and boronic acid, and similar molecules having an affinity which permits their association in a binding assay. Furthermore, binding pairs can include members that are analogs of the original binding member, for example an analyte-analog or a binding member made by recombinant techniques or molecular engineering. If the binding member is an immunoreactant it can be, for example, an antibody, antigen, hapten, or complex thereof, and if an antibody is used, it can be a monoclonal or polyclonal antibody, a recombinant protein or antibody, a chimeric antibody, a mixture(s) or fragment(s) thereof, as well as a mixture of an antibody and other binding members. The details of the preparation of such antibodies, peptides and nucleotides and their suitability for use as binding members in a binding assay are well-known to those skilled-in-the-art.

The term "analyte" or "analyte of interest", as used herein, refers to the compound or composition to be detected or measured and which has at least one epitope or binding site. The analyte can be any substance for which there exists a naturally occurring binding member or for which a binding member can be prepared. Analytes include, but are not intended to be limited to, toxins, organic compounds, proteins, peptides, microorganisms, amino acids, carbohydrates, nucleic acids, hormones, steroids, vitamins, drugs (including those administered for therapeutic purposes as well as those administered for illicit purposes), virus particles and metabolites of or antibodies to any of the above substances. For example, such analytes include, but are not intended to be limited to, ferritin; creatinine kinase MIB (CK-MB); digoxin; phenytoin; phenobarbitol; carbamazepine; vancomycin; gentamycin; theophylline; valproic acid; quinidine; leutinizing hormone (LH); follicle stimulating hormone (FSH); estradiol, progesterone; IgE antibodies; vitamin B2 micro-globulin; glycated hemoglobin (Gly. Hb); cortisol; digitoxin; N-acetylprocainamide (NAPA); procainamide; antibodies to rubella, such as rubella-lgG and rubella-lgM; antibodies to toxoplasmosis, such as toxoplasmosis IgG (Toxo-lgG) and toxoplasmosis IgM (Toxo-lgM); testosterone; salicylates; acetaminophen; hepatitis B virus surface antigen (HBsAg); antibodies to hepatitis B core antigen, such as anti hepatitis B core antigen IgG and IgM (Anti-HBC); human immune deficiency virus 1 and 2 (HTLV); hepatitis B e antigen (HBeAg); antibodies to hepatitis B e antigen (Anti-HBe); thyroid stimulating hormone (TSH); thyroxine (T4); total triiodothyronine (Total T3); free triiodothyronine (Free T3); carcinoembryoic antigen (CEA); and alpha fetal protein (AFP); and drugs of abuse and controlled substances, including but not intended to be limited to, amphetamine; methamphetamine; barbiturates such as amobarbital, secobarbital, pentobarbital, phenobarbital, and barbital; benzodiazepines such as librium and valium; cannabinoids such as hashish and marijuana; cocaine; fentanyl; LSD; methapualone; opiates such as heroin, morphine, codeine, hydromorphone, hydrocodone, methadone, oxycodone, oxymorphone and opium; phencyclidine; and propoxyhene. The term "analyte" also includes any antigenic substances, haptens, antibodies, macromolecules and combinations thereof.

The term "analyte-analog", as used herein, refers to a substance which cross-reacts with an analyte-specific binding member, although it may do so to a greater or a lesser extent than does the analyte itself. The analyte-analog can include a modified analyte as well as a fragmented or synthetic portion of the analyte molecule, so long as the analyte-analog has at least one epitopic site in common with the analyte of interest. An example of an analyte-analog is a synthetic peptide sequence which duplicates at least one epitope of the whole-molecule analyte so that the analyte-analog can bind to an analyte-specific binding member.

The term "magnetically-labeled reagent", as used herein, refers to a substance involving a magnetically-attractable label attached to a binding member. The attachment may be affected by covalent or non-covalent binding means, linking arms, and the like. However, the method of attachment is not critical to the present invention. Upon the application of a magnetic field, the magnetically-attractable label allows the reagent to produce a detectable response that will be directly or indirectly related to the amount of analyte in the test sample. The binding member component of the reagent may be selected to directly bind the analyte or to indirectly bind the analyte by means of an ancillary specific binding member, which is described in greater detail hereinafter. Magnetically-labeled reagents may be attached to ancillary specific binding members before, during or after contacting the magnetically-labeled reagent with the test sample and/or other assay reagents. The terms "binding member attached to a magnetically-attractable particle", "binding member attached to a magnetic material", "binding member attached to a magnetic label", "binding member attached to a magnetically-responsive label" and similar terms are interchangeable and are used to refer to the main characteristic of the magnetically-labeled reagents of the present invention, i.e., the label produces a detectable response when placed in the vicinity of a magnetic field.

The term "solid phase", as used herein, refers to any material to which analyte, analyte complexes or assay reagents become bound and from which unreacted assay reagents, test sample or test solutions can be separated. The solid phase generally has a binding member

immobilized on or in its surface to form a "solid-phase reagent", that allows the immobilization of the analyte, the magnetically-labeled reagent or another assay reagent. Binding members which are immobilized in or on the solid phase may be selected to directly bind the analyte or to indirectly bind the analyte by means of an ancillary specific binding member which can be immobilized to the solid-phase reagent before, during, or after contacting the solid-phase reagent with the test sample and/or other assay reagents.

It will be understood, of course, that the solid phase may comprise multiple components and that the immobilized binding member can be bound directly to any or all components of the solid phase. For example, a multiple component solid phase can include a solid-phase reagent that is physically entrapped or retained and immobilized within a second or supplementary component of the solid phase by a physical, chemical or biochemical means. As a further example, an analyte-specific binding member can be attached to insoluble microparticles which are subsequently retained by a porous material. By "retained" it is meant that the microparticles, once on the porous material, are not capable of substantial movement to positions elsewhere within the porous material. A first solid phase component, which itself can be a solid-phase reagent, can be retained by a supplementary component of the solid phase before, during or after contacting the first solid phase component with the test sample and/or other assay reagents. In most embodiments, however, the binding member is bound or attached to a single solid phase component prior to contacting the thusly formed solid-phase reagent with the test sample or other assay reagents.

The term "ancillary binding member", as used herein, refers to any member of a binding pair which is used in the assay in addition to the binding members of the magnetically-labeled reagent or solid-phase reagent. For example, in instances where the analyte itself cannot directly attach to the magnetically-labeled reagent, an ancillary binding member can be capable of binding the magnetically-labeled reagent to the analyte of interest. As it will be understood, of course, one or more ancillary binding members can be used in an assay and such ancillary binding member(s) can be attached to the magnetically-labeled reagent or solid-phase reagent either before, during or after the magnetically-labeled reagent or solid-phase reagent is contacted with a test sample or other assay reagent. The ancillary binding member can be incorporated into the assay device or it can be added to the device as a separate reagent solution.

DESCRIPTION OF THE INVENTION

When a magnetically-responsive material is placed under the influence of a magnetic field, the material will tend to move toward or away from the region where the magnetic field is the strongest. For example, a paramagnetic material, such as ferrite, will be attracted to the magnetic field while a diamagnetic material, such as polystyrene, will move away from the magnetic field. The extent of the response of such magnetically-responsive materials can be used as a measure of the amount of material present. The present invention results from the unexpected and surprising discovery that, when a magnetically-responsive material-is used as a label in a binding assay, it is possible to detect the presence or amount of either or both of the free or bound label by measuring the extent of the response resulting from the label's reaction to an applied magnetic field. The magnetic label's response to a magnetic field can manifest itself in ways such as, for example, a detectable movement of the magnetically-responsive material or a detectable resultant force exerted by the magnetically-responsive material. Furthermore, the strength of the force or the extent of movement bears a definite relationship to the amount of the bound or free magnetically-attractable label which thereby permits a determination of the presence or amount of an analyte in the test sample.

Conventional heterogeneous binding assay formats require vigorous washing of the solid phase to separate bound and unbound labeled reagent and to suppress the nonspecific binding of materials to the solid phase. Such wash steps complicate the assay protocol and restrict the assay to the use of binding pair members having high affinity, i.e., a binding strength that will withstand such physical manipulation. Conversely, the present invention avoids the need for complex washing steps in binding assays because unbound or non-specifically bound label can be separated from the reaction mixture by the application of a first magnetic field prior to the detection of specifically bound label by means of a second magnetic field. The high degree of control that is possible over the magnetic field permits the use of a first field that is suitable to separate free or non-specifically bound label from a reaction mixture without affecting specifically bound label. In turn, this permits the use of lower affinity binding members whose binding will not be significantly affected by the first magnetic field.

In conventional particle agglutination assays, binding members of low affinity can be used because several binding sites on each member can cooperate to give high avidities, and the absence of wash steps allows weak associations to be maintained while simplifying the assay format. Signal amplification results from the fact that the interaction of a few binding sites can cause the aggregation of binding members several orders of magnitude greater in size and mass than the original members, and thereby provide a macroscopic change which can be interpreted visually. However, particle agglutination assays are often difficult to interpret, do not yield quantitative results, and are not readily amenable to automation.

The present invention solves the aforementioned problems of conventional heterogeneous and agglutination assays by placing the magnetic label in a magnetic field, and measuring the consequences of the magnetic force exerted upon the label to provide a qualitative or quantitative assay readout. The force affect of the magnetic field upon the magnetic label enhances the detection of the captured or aggregated magnetic label while suppressing non-specific interference from any non-magnetic substances. For example, the nonspecific binding of extraneous substances to the solid phase will not interfere with the analyte determination because the force affect of the magnetic label in a magnetic field is measured, as opposed to determining the total weight of the resulting binding complex or reagents adhering to the solid phase. Force enhancements approaching about three orders of magnitude have been achieved by the application of a magnetic field and the detection of the resultant force affect on the magnetic label in that field. In comparison to the detection of weight changes due to binding reactions (e.g., the detection of the weight of bound analyte as is determined by conventional gravimetric analyses), the present invention provides binding

assay signal enhancements of about nine orders of magnitude and is sufficient to detect analyte concentrations in the femtomolar (10^{-15} mole or one quadrillionth mole) range. In this regard, small levels of force can be readily determined using detection means which include, but are not intended to be limited to, electronic balances; optical sensors; piezoelectric pressure sensing devices such as, for example, micromechanical silicon devices or electronic chips; vibrating fiber devices; coils which produce a field which is disrupted by the presence of a magnetically-responsive label such as, for example induction coils and the like; and cantilever beam devices including, but not intended to be limited to those used to sense force changes in an atomic force microscope; and the like. This enables very sensitive assays and obviates the need for amplification of the label as required in many conventional assays.

According to the present invention, the intensity of the magnetic field can be precisely manipulated by, for example, means of an electromagnetic or a movable permanent magnet. A field intensity can be chosen which is optimal for a particular assay and particular binding reagents, such that a field sufficient to remove unbound and non-specifically bound magnetic label can be applied without disrupting the associations formed between the binding members. This provides the opportunity to use binding members having lower binding affinities than those typically found in binding assays.

It is to be understood that the aforementioned advantages permit the assays to be readily adapted to computer control. In addition, the intensity of the magnetic field can be precisely manipulated to disrupt the associations formed between the binding members. Thus, the present invention also provides a means to evaluate the binding affinities or association constants of binding members.

(a) Assay Reagents

The selection of a particular composition of magnetic label material is not critical to the present invention. Preferably, the magnetically-attractable material can bind, carry or be modifiable so as to attach to a binding member which will in turn bind another assay reagent or a component present in a test sample. It is also preferred that the label be magnetically attractable to an extent which permits partitioning of the bound and unbound magnetically-labeled reagent and the production of a detectable response upon exposure to a magnetic field. For the purposes of the present invention, a material is magnetically responsive if it is influenced by the application of a magnetic field, such as, for example, if it is attracted or repulsed or has a detectable magnetic susceptibility or induction. A variety of different magnetically-labeled reagents can be formed by varying either the label component or the binding member component of the reagent. It will be understood, of course, that the choice involves consideration of the analyte to be detected and the desired optimization of the assay technique.

A wide variety of magnetically-attractable materials which are suitable for use as magnetic labels are commercially available or the production techniques therefor are well known in the art. The preferred characteristics of the magnetically-attractable label can be achieved by a wide variety of magnetic materials. Magnetically-attractable materials include, but are not intended to be limited to, ferromagnetic, ferrimagnetic, paramagnetic, superparamagnetic materials, and the like. The term "ferromagnetic" is generally used to describe materials which are attracted to a magnet and which typically become permanently magnetized upon exposure to a magnetic field. Ferromagnetic materials may also be reduced in particle size such that each of the particles is a single domain. In this state of subdivision, the ferromagnetic material may be referred to as "superparamagnetic", and characterized by the absence of any permanent measurable magnetization. Suitable magnetically-repulsed materials include, but are not intended to be limited to, diamagnetic materials such as, for example, organic polymers, including polystyrene, and the like.

Suitable ferromagnetic, ferrimagnetic, paramagnetic and superparamagnetic materials include, but are not intended to be limited to, metals such as iron, nickel, cobalt, chromium, manganese and the like; lanthanide series elements such as neodymiun, erbium and the like; alloys such as magnetic alloys of aluminum, nickel, cobalt, copper and the like; oxides such as ferric oxide (Fe_3O_4), γ-ferric oxide (γ-Fe_3O_4), chromium oxide (CrO_2), cobalt oxide (CoO), nickel oxide (NiO_2), manganese oxide (Mn_2O_3) and the like; composite materials such as ferrites and the like; and solid solutions such as magnetite with ferric oxide and the like. Preferred magnetic materials are magnetite, ferric oxide (Fe_3O_4) and ferrous oxide (Fe_2O_3).

Suitable particle compositions include, but are not intended to be limited to, those particle types referred to in Table 1.

TABLE 1

TYPE	FORM	COMPOSITION
Solid particles		iron
		iron oxide
		core of magnetic material, coated with a metal oxide
		colloidal magnetic particles containing magnetite or hematite and having a specific gravity of up to 8 and size range of less than 1 to 800 nanometers
Layered particles	magnetic material core with a nonmagnetic coating	a magnetic metal oxide core generally surrounded by a polymeric silane coat
		a water-insoluble metal substrate coated with a condensation product of an aminobenzoic acid with an aldehyde, suitable for coupling to a compound having biological affinity
	magnetic material core with a nonmagnetic coating	a core formed of a single particle of magnetically-responsive material with a coating of a water-insoluble cross-linked polymeric material having reactive groups at the surface thereof
Layered particles (cont.)	nonmagnetic core with a magnetic material coating	an organic polymer particle with a ferrite coating
		a sphere of thermoplastic material with a magnetic material coating (on at least a portion of the core surface)
		a metal-coated polyaldehyde microsphere
		an inner core polymer particle (e.g., polystyrene) with a magnetically-responsive metal oxide/polymer coating evenly covering the inner core
	nonmagnetic core with a	an agarose-encapsulated metal-coated polyaldehyde microsphere

TABLE 1-continued

TYPE	FORM	COMPOSITION
	magnetic material layer and a nonmagnetic coating	a thermoplastic resin bead (e.g., polystyrene, polyvinyl chloride, polyacrylate, nylon, etc.) with from 1–25% by weight of magnetically-responsive powder bound on the bead, and a polymer coated thereon having functional groups to bind a biologically active component
Composite particles	magnetic material embedded within a nonmagnetic material	iron-containing magnetic crystals (<1000 Å) incorporated within a glass and/or crystal structure
Composite particles (cont.)	magnetic material embedded within a nonmagnetic material	a copolymer matrix: 30–99% by weight, of at least one monoethylenic monomer which does not coordinate with a metal complex, 0.5–50% by weight, of at least one crosslinkable polyethylenic monomer which does not coordinate with a metal complex, and 0.5–30% by weight, of at least one nucleophilic monomer which can be coordinated with a metal complex, with encapsulated crystalites of a metal magnetizable particles of a size less than 300 Å, encapsulated in an organpolysiloxane matrix
		a particulate reaction product of a water-soluble form of iron and a water-soluble polymer having available coordination sites (free electron pair for a coordinate bond with a transition metal atom)
		an organic, inorganic or synthetic polymer matrix containing a magnetically-attractable material
		magnetizable particles of a size less than 300 Å, encapsulated in an organpolysiloxane matrix
		a particulate reaction product of a water-soluble form of iron and a water-soluble polymer having available coordination sites (free electron pair for a coordinate bond with a transition metal atom)
Composite particles (cont.)	magnetic material embedded within a nonmagnetic material	an organic, inorganic or synthetic polymer matrix containing a magnetically-attractable material
		a continuous phase of a water-insoluble polymeric matrix having dispersed (embedded) therein: a magnetically attractable material, and a particulate absorbent material (selected from charcoal, talc, ion exchange resins, Fuller's earth, silicon dioxide, oxides of zirconium or aluminum or titanium, porous glass, zeolites, natural or synthetic polymers, polymerized first or second antibodies or polymerized enzymes, cell surface antigens or receptors in a particulate form, subcellular particles and bacterial cells)
		particles made by polymerizing one or more monomers in the presence of magnetically-attractable solids to form directly a synthetic water-insoluble polymeric matrix having the solids uniformly embedded therein
		particles of cross-linked protein or polypeptide and a magnetically -responsive material made by combining: an organic solvent solution of a high MW polymer (e.g., polystyrene), a particulate magnetically-responsive material and a polyfunctional cross-linking agent (e.g., polyaldehyde)
Matrix particles	magnetic material dispersed within a nonmagnetic material	hydrophobic vinyl aromatic polymer particles having a mean diameter between 0.03 and 5 microns and a magnetically-charged material in an amount from 0.5 to 50% by weight with respect to the polymer portion of the particles, the magnetically-charged material being dispersed within the polymer particles
		a filler selected from the group consisting of a metal, metal alloy, metal oxide, metal salt, metal sulfide, pigment and metallic chelate compound, and an oleophilic surface layer upon the filler, and a layer of polymer upon the oleophilic-surfaced filler

Magnetic labels formed as matrix or composite particles may optionally include additional coatings or layers of magnetic or nonmagnetic materials or mixtures thereof. Matrix compositions can be made by any suitable means including, but not intended to be limited to, the polymerization of the magnetically-attractable material with the selected monomer, the swelling of the matrix material with the introduction of the magnetically-attractable material into pores within the matrix, and the like. The matrix can include, for example, organic and inorganic materials such as glass, cellulose, synthetic polymer materials, agarose, and the like. Suitable polymer materials include, but are not intended to be limited to, polymers of styrene; substituted styrenes; naphthalene derivatives; acrylic and methacrylic acids; acrylamide and methacrylamide; polycarbonate; polyesters; polyamides; polypyrrole; aminoaromatic acids; aldehydes; proteinaceous materials such as gelatin, albumin and the like; polysaccharides such as starch, dextran and the like; and copolymers of polymeric materials. The polymer may also be used in an admixture with an inert filler or may include an absorbent material.

Generally, the magnetic particles used in the present invention are substantially spherical in shape, although other shapes are suitable and may be advantageous in some circumstances. Other possible shapes include, but are not intended to be limited to, plates, rods, bars and irregular shapes. The diameter of the magnetic label can preferably range from between about 0.01 microns (μm) and about 1,000 μm, more preferably from between about 0.01 μm and about 100 μm, and most preferably from between about 0.01 μm and about 10 μm. As it will be appreciated by those skilled in the art, the composition, shape, size, and density of the magnetically-attractable material may vary widely and a label can be

selected based upon such factors as the analyte of interest and the desired assay protocol.

According to one embodiment of the present invention, the magnetic particles can be selected to have a specific gravity so as to tend to be suspended within the reaction mixture thereby enhancing the reactivity of the binding member. Generally, small magnetic particles with a mean diameter of less than about 0.03 μm (300 Å) can be kept in solution by thermal agitation and do not spontaneously settle. In alternative embodiments, the magnetic particles can be selected to have a specific gravity so as to tend to settle in the reaction mixture thereby enhancing the reactivity of the binding member with the immobilized reagent on the solid phase. Generally, large magnetic particles having a mean diameter greater than about 10 microns can respond to weak magnetic fields. Although large or dense labels may be used, such labels may require that the reaction mixture be stirred or agitated during the incubation steps to inhibit settling of the particles. In another embodiment, the magnetic particles can be selected to remain dispersed in the reaction mixture for a time sufficient to permit the required binding reactions without the need for a stirring or mixing means.

In forming the magnetically-labeled reagent, the attachment of the binding member to the magnetically-attractable material may be achieved by any suitable attachment or coupling means including, but not intended to be limited to, adsorption, covalent bonding, cross-linking (chemically or through binding members), a combination of such attachment means, and the like. Typically, coupling groups and coupling or linking agents are selected so that the binding activity of the binding member is not substantially modified or destroyed upon attachment to the label. The quantity of binding member which may be attached to the magnetically-attractable label is largely dependent upon its concentration, the conditions used, and the amount of and nature of the available functional groups on the magnetically-attractable label or coupling agent.

Preferably, the binding member is covalently bonded to the magnetically-attractable label, and the covalent bond may be formed between one component and a chemically active form of the other component. For example, an active ester such as N-hydroxysuccinimide can be introduced into one component and allowed to react with a free amine on the other component to form a covalent coupling of the two. Other examples include, but are not intended to be limited to, the introduction of maleimide to one component which is then allowed to react with endogenous or introduced sulfhydryl moieties on the other component; the oxidation of endogenous or introduced carbohydrate groups on one component to form aldehydes which can react with free amines or hydrazides on the other component; where the magnetically-attractable label includes a polymer coating or matrix, the polymer may be selected so that it contains, or can be provided with, suitable reactive groups such as, for example, azide, bromoacetyl, amino, hydroxyl, sulfhydryl, epoxide, carboxylic or other groups to facilitate the attachment of the binding member; and the like. Suitable reagents as well as conjugation techniques for synthesizing the magnetically-labeled reagent are well-known to those skilled-in-the-art. It will be understood, of course, that the methods of synthesizing a magnetically-labeled reagent are not intended to limit the invention.

The solid phase material and solid-phase reagents can generally comprise nonporous materials including, but not intended to be limited to, polymers such as, for example, styrene; substituted styrenes; naphthalene derivatives; acrylic and methacrylic acids; acrylamide and methacrylamide; polycarbonate; polyesters; polyamides; polypyrrole; polypropylene; latex; polytetrafluoroethylene; polyacrylonitrile; polycarbonate; glass or similar materials; aminoaromatic acids; aldehydes; proteinaceous materials such as gelatin, albumin and the like; polysaccharides such as starch, dextran and the like; and copolymers of polymeric materials. Such materials can take the form of particles, beads, tubes, slides, tapes, webbing, plates or wells. Thus, the solid phase can be the "reaction vessel" in which the binding assay takes place. For example, the solid phase can be a microtitre well, or it can be a material or materials contained within the reaction vessel, such as, for example, a bead within a test tube.

The solid phase material can also be any suitable chromatographic, bibulous, porous or capillary material. Accordingly, the solid phase material can include, but is not intended to be limited to, a fiberglass, cellulose or nylon pad for use in a flow-through assay device having one or more layers containing one or more of the assay reagents; a dipstick for a dip and read assay; a paper, nitrocellulose or glass fiber test strip for chromatographic or thin layer chromatographic techniques in which one or all of the reagents are contained in separate zones of a single strip of solid phase material; or any absorbent material well known to those skilled in the art.

As further examples, natural, synthetic or naturally occurring materials that are synthetically modified, can be used as a solid phase material. Examples of such materials include, but are not intended to be limited to, polysaccharides such as cellulose materials including paper and the like and cellulose derivatives such as cellulose acetate and nitrocellulose; silica; silicon particles; inorganic materials such as deactivated alumina, or other inorganic finely divided material uniformly dispersed in a porous polymer matrix, with polymers such as vinyl chloride, vinyl chloride polymer with propylene, and vinyl chloride polymer with vinyl acetate; naturally occurring and synthetic cloth such as cotton, nylon and the like; porous gels such as silica gel, agarose, dextran, gelatin and the like; polymeric films such as polyacrylates and the like; protein binding membranes; and the like. The solid phase may also comprise microparticles which can be selected by one skilled in the art from any suitable type of material including, but not intended to be limited to, polystyrene, polymethylacrylate, polyacrylamide, polypropylene, latex, polytetrafluoroethylene, polyacrylonitrile, polycarbonate, glass, and the like.

While the solid phase material preferably has a reasonable strength, or such strength can be provided by means of a support, it preferably does not interfere with the production of a detectable signal. It will be understood, of course, that the solid-phase material is typically nonmagnetic or, if magnetic, that its magnetic contribution to the assay is correctable by, for example, positioning such material in a manner where it is not affected by a magnetic field or such material can be demagnetized. The means of attaching a binding member to a solid phase to thereby form a solid-phase reagent, encompasses both covalent and non-covalent means which have been outlined previously with regard

to synthesizing a magnetically-labeled reagent. It is generally preferred that the binding member be attached to the solid phase by covalent means.

(b) Assay Methods & Devices

The methods and devices of the present invention may be applied to any suitable assay format involving binding pair members including, but not limited to, those binding members described above. The assay methods of the present invention utilize the response of a magnetically-labeled reagent to the influence of a magnetic field to qualitatively or quantitatively measure binding between binding pair members. According to the present invention, the presence of an analyte mediates whether or not the magnetically-labeled reagent becomes immobilized to a solid-phase reagent. The analyte can thereby control the extent of the magnetically-labeled reagent's response to the influence of a magnetic field. Hence, by measuring the magnetically-labeled reagent's response to the magnetic field, the presence or amount of analyte contained in a test sample can accurately be determined.

The present invention is applicable to various competitive assay formats and sandwich assay formats well known in the art. Various competitive, inhibition and sandwich assays have been described whereby a labeled reagent is partitioned between a liquid phase and a solid phase in relation to the presence of the analyte in the test sample. According to a competitive assay format, a magnetically-labeled reagent can comprise a first binding member (an analyte analog) attached to a magnetically-attractable label, to thereby form a magnetically-labeled reagent. A solid-phase reagent can comprise a second binding member, which is specific for the analyte and analyte analog, attached to a solid phase. During the course of the assay, an analyte in the test sample and the magnetically-labeled analyte analog compete for binding sites on the solid-phase reagent. Alternatively, the binding member attached to the solid phase may be an analyte-analog selected to compete with the analyte for binding to a magnetically-labeled binding pair member. Hence, the quantity of magnetically-labeled reagent that becomes bound to the solid phase is inversely proportional to the amount of analyte in the test sample.

According to a sandwich assay format, a first binding member is attached to a magnetically-attractable label to form a magnetically-labeled reagent and a second binding member is attached to the solid phase to form a solid-phase reagent. The binding members are selected to directly or indirectly bind the analyte of interest. During the course of the assay, the magnetically-labeled reagent becomes immobilized to the solid-phase reagent by binding the analyte that has bound the solid-phase reagent. Thus, the quantity of magnetically-labeled reagent that becomes bound to the solid-phase reagent is directly proportional to the amount of analyte in the test sample.

According to the present invention, assay protocols may optionally comprise the use of ancillary binding members to indirectly bind the analyte or analyte analog to the magnetically-labeled reagent or to the solid-phase reagent. The ancillary binding member can be attached to a solid-phase reagent or magnetically-labeled reagent before, during or after contacting the solid-phase reagent or magnetically-labeled reagent with the test sample or other assay reagents. In addition, the assay protocols may comprise, for example, contacting the assay reagents and test sample simultaneously to form a reaction mixture, or the assay reagents and test sample can be contacted sequentially, and for a time period suitable for binding to form multiple reaction mixtures.

According to such assay protocols, after a period suitable for binding, the unbound magnetically-labeled reagent can be separated from the bound magnetically-labeled reagent. It will be understood, of course, that the separation of bound and unbound magnetically-labeled reagent may involve the complete removal of the unbound magnetically-labeled reagent from the reaction mixture and/or from that magnetically-labeled reagent which is immobilized to the solid-phase reagent. The separation of bound and unbound magnetically-labeled reagent may also involve the segregation of the unbound magnetically-labeled reagent from the immobilized magnetically-labeled reagent such that the unbound magnetically-labeled reagent remains in the reaction mixture but does not significantly produce a detectable response when the bound magnetically-labeled reagent is placed in the vicinity of a magnetic field. Alternatively, either the unbound or bound magnetically-labeled reagent can be observed for a response to a magnetic field. Further, both the unbound and bound magnetically-labeled reagents can be observed for a response to a magnetic field whereby a ratio of the partitioning can be observed.

Generally, devices according to the present invention comprise components for performing magnetically assisted binding assays as taught herein. Accordingly, such devices preferably comprise (i) a reaction vessel; (ii) a separation means for separating the immobilized magnetically-labeled reagent from the unbound magnetically-labeled reagent; (iii) a magnetic field generator means for the application of a magnetic field to the magnetically-labeled reagent; and (iv) a measurement means to assess the effect of the magnetic field generated by the magnetic field generator means on the magnetically-labeled reagent as a measure of the presence or amount of analyte in the test sample.

The reaction vessel can be anything capable of containing the assay reagents disclosed herein and where unbound and immobilized magnetically-labeled reagent can be produced in relation to the amount of an analyte in a test sample. The reaction vessel can comprise any material previously described herein with respect to the solid phase. Moreover, the solid phase or solid-phase reagent can serve as the reaction vessel such as, for example, test-tubes, microtiter wells, tubing, slides and the like.

Separating the bound magnetically-labeled reagent from the unbound magnetically-labeled reagent can be accomplished by any means suitable for partitioning the unbound and bound magnetically-labeled reagent. For example, a vibratory or tilting means could be used to effectuate the separation. Preferably, the magnetically-labeled reagent that is not immobilized to the solid-phase reagent is separated from the solid phase by the application of a magnetic field which is sufficient to move unbound magnetically-labeled reagent, but not the bound magnetically-labeled reagent. For example, the unbound magnetically-labeled reagent may be removed from the reaction mixture by inserting a magnetic probe into the reaction mixture and then removing the probe with any unbound magnetically-labeled reagent that is attracted to that probe. In another embodiment, the unbound magnetically-labeled reagent may be

pulled from the reaction mixture by placing a magnet outside of the reaction vessel and moving the magnet along the vessel bottom and/or wall, thereby pulling the unbound magnetically-labeled reagent from the reaction mixture or away from the reagent which is immobilized to the solid phase. In yet another embodiment, a magnetic means may be brought into proximity with the surface of the reaction mixture such that unbound magnetically-labeled reagent is sequestered at the air/liquid interface of the reaction mixture, thereby separating unbound magnetically-labeled reagent from the immobilized reagent. In a further embodiment, the unbound reagent can be moved away from the immobilized magnetically-labeled reagent, and retained in a suitable manner, such that the unbound reagent is retarded from moving back to the site of the immobilized magnetically-labeled reagent.

The magnetic field generator means can be any means for generating a magnetic field which elicits a response from the magnetically-labeled reagent. Preferred magnetic field generator means include permanent magnets and electromagnets. It will also be understood, of course, that the magnetic field generator means may also be used to separate the unbound or free magnetically-labeled reagent from the bound or immobilized reagent.

A magnetically-labeled reagent's response to a magnetic field can be manifested in many measurable forms including a resulting force or movement of the reagent such as, for example, an apparent weight change of the reagent, a displacement of the reagent, a mass change of the reagent, and the like. It will be understood, of course, that these manifestations can be measured directly by detecting the manifestations of the magnetically-labeled reagent, or the manifestations can be measured indirectly by detecting the magnetically-labeled reagents effect on, for example, the solid phase, the solid-phase reagent or the magnetic field generator means. The influence of the magnetic field upon a magnetically-labeled reagent may be observed or detected and measured by any means suitable for directly or indirectly measuring the magnetically-labeled reagent's response to the magnetic field. For example, a change in the apparent weight can be detected and measured by a balance; a change in apparent mass can be detected and measured by a balance or a resultant change in frequency of a quartz crystal; a displacement can be detected and measured by an optical sensor means to assess the magnitude of a change from an initial position to a subsequent position assumed by the magnetically-labeled reagent, solid-phase reagent or solid phase; a movement can be detected and measured by motion detection means to assess movement, such as, for example, a piezoelectrical film, or a coil such as, for example, a susceptometer which can create a field that is measurably disrupted by the presence and/or movement of magnetic material; and a change in the amount of stress can be detected by incorporating stress sensitive materials into a vessel or solid-phase material such that upon the application of a magnetic field the change in stress would be detectable. It will be understood, of course, that depending upon the particular assay, it may be preferred to detect, directly or indirectly, the unbound magnetically-labeled reagent's, the bound magnetically-labeled reagent's or both the bound and unbound magnetically-labeled reagent's response to the magnetic field. It will also be understood, of course, that a wide variety of instruments can be used to detect mass changes, position changes, movements, weight changes, force changes, magnetic susceptibility, induction and the like; all of which result from the interaction between a magnetic field and the magnetically-labeled reagent.

While various devices and assay protocols are contemplated by the present invention, the following protocols represent examples, and are not intended to be limited to, two sandwich assay formats using the magnetically assisted detection of a magnetically-labeled reagent of the present invention. In this regard, the following protocols, and protocols contemplated by the present invention, can be performed in any order of steps or, alternatively, in a simultaneous manner.

Protocol A

1) a first binding member selected to bind the analyte is attached to a magnetically-attractable label to form a magnetically-labeled reagent;
2) a second binding member selected to bind a second binding site on the analyte is attached to a solid phase to form a solid-phase reagent;
3) a test sample is contacted with the solid-phase reagent to form a first reaction mixture whereby the analyte becomes bound to the solid-phase reagent;
4) the first reaction mixture is contacted with the magnetically-labeled reagent to form a second reaction mixture whereby the magnetically-labeled reagent becomes immobilized upon the solid-phase reagent by binding the captured analyte (the proportion of magnetically-labeled .reagent that becomes bound to the solid-phase reagent is directly related to the amount of analyte in the test sample);
5) the unbound magnetically-labeled reagent is removed from the second reaction mixture;
6) the solid-phase reagent and the magnetically labeled reagent bound thereto is placed on a detection means;
7) the second reaction mixture is exposed to a magnetic field such that a magnetic force is exerted on the magnetically-labeled reagent immobilized upon the solid phase, the influence of this force is manifested by the captured magnetically-labeled reagent and the degree of the manifestation is determined by the detection means; and
8) the measurable degree of the manifestation provides a measure of the quantity of the magnetically-labeled reagent bound to the solid phase.

Protocol B

1) a first binding member selected to bind the analyte is attached to a magnetically-attractable label to form a magnetically-labeled reagent;
2) a second binding member selected to bind a second binding site on the analyte is attached to a solid phase to form a solid-phase reagent;
3) a test sample is contacted with the solid-phase reagent to form a first reaction mixture whereby the analyte becomes bound to the solid-phase reagent;
4) the first reaction mixture is contacted with the magnetically-labeled reagent to form a second reaction mixture whereby the magnetically-labeled reagent becomes immobilized upon the solid-phase reagent by binding the captured analyte (the proportion of magnetically-labeled reagent that becomes bound to the solid-phase reagent is directly related to the amount of analyte in the test sample);

5) the solid-phase reagent and magnetically labeled reagent are placed on a detection means;

6) the second reaction mixture is exposed to a magnetic field such that a magnetic force is exerted on the magnetically-labeled reagent, and the influence of the magnetic field causes the unbound magnetically-labeled reagent to manifest the effect of the magnetic field which is detected by the detection means; and

7) the degree of the manifestation provides a measure of the quantity of the bound magnetically-labeled reagent.

FIG. **1** and FIG. **2** illustrate a schematic view of the magnetically assisted measurement of the binding of a magnetically-labeled reagent to a solid phase, and substantially follows Protocol A after the unbound magnetically-labeled reagent has been removed from the second reaction mixture and the solid phase has been placed upon a detection means (step **6**). As shown by FIG. **1**, the solid-phase reagent comprises a well (**20**) that contains the immobilized magnetically-labeled reagent (**10**). The well is introduced to the detection means by setting it upon or affixing it to a support means (**30**). The support means rests upon a balance means (**50**). On a typical top loading microbalance, the balance has a pan (**40**) which will receive the support, and once the balance receives the support, or once the support receives the well, the balance can be tared or set to equilibrium (zeroed).

In FIG. **2**, a magnet (**60**) is positioned into or brought into proximity with the vicinity of the well, whereby the magnetic field exerts a force upon the magnetically-labeled reagent immobilized to the solid-phase reagent. The force exerted upon the magnetically-labeled reagent is manifested as an apparent change in the weight of the solid phase which is registered on the scale of the balance means (**50**). Generally, the magnet is affixed to an arm means (**70**) which allows delicate adjustments of the movement of the magnet toward and away from the solid phase.

The magnetic field may be provided by means of a permanent magnetic or an electromagnet and may be applied intermittently or continuously. An electromagnetic may be used so that the magnetic field can be turned off and on rather than moving the magnet or the solid phase. An electromagnet can also be computer controlled, thereby providing for fine adjustments to magnetic field strength. Furthermore, an electromagnet can be used to generate an alternating magnetic field which can have the further advantage of causing the mixing of the magnetically-labeled reagent in the reaction mixture if such mixing is desired.

FIG. **3** and FIG. **4** illustrate a schematic view of an alternate means for the measurement of the binding of a magnetically-labeled reagent to the solid phase, and substantially follows Protocol A after the unbound magnetically-labeled reagent has been removed from the solid phase and the solid phase has been placed on detection means (step **6**). As shown in FIG. **3**, the solid-phase reagent comprises a strip of bendable material (**25**) which has immobilized magnetically-labeled reagent (**10**) thereon. The bendable material is supported on a foundation means (**45**). The detection means includes a cantilever beam (**35**) which contacts the bendable material and deflects in response to any movement of the solid phase. Detection is accomplished by means of a laser light source (**80**) and an optical sensor means (**50**). Coherent light from the laser is reflected from the cantilever beam onto the optical sensor. Any deviation in the position of the cantilever beam results in a shift of position or deflection of the reflected light striking the optical sensor, thereby causing a change in its output. The greater the distance traveled by the light, the greater the sensitivity of such a measurement means.

In FIG. **4**, a magnet (**60**) is positioned in proximity to the bendable material, whereby the magnetic field exerts a force upon the magnetically-labeled reagent immobilized on the bendable material. The force exerted upon the bendable material will cause a displacement of the material or a distortion in the shape of the material. The degree of displacement or distortion from the original position of the bendable material is largely dependent upon the amount of magnetically-labeled reagent bound to the solid phase and can be measured by the detection means.

FIG. **5***a* and FIG. **5***b* illustrate schematic views of yet another means for the determination of the binding reaction by detecting a force exerted by the unbound magnetically-labeled reagent as a consequence of being exposed to magnetic field. Additionally, the Figures substantially correspond to Protocol B after the solid phase has been placed on the detection means (step **5**). As seen in FIG. **5***a*, the solid phase comprises a well (**10**) that contains magnetically-labeled reagent (**20**), at least a portion of which is immobilized in the well. The well is then set upon or affixed to a balance means (**50**) having a pan (**40**) which receives the well. Once the balance receives the well, the balance can be zeroed. In FIG. **5***b*, a magnet (**60**) is positioned into or brought into proximity with the vicinity of the surface of the well contents whereby the magnetic field exerts a force upon the magnetically-labeled reagent. Under the influence of this force the unbound magnetically-labeled reagent (**25**) migrates to the air-liquid interface where the magnetic attraction is more intense due to the closer proximity of the magnet. The bound magnetically-labeled reagent resists movement under this level of magnetic field intensity and remains bound through the analyte to the well bottom. The unbound magnetically-labeled reagent at the air-liquid interface strains upward against the surface tension of the liquid surface, causing a change in the apparent weight of the solid phase which is registered as a change of the readout on the scale of the balance means (**50**). As the magnet is moved closer to the well, the increased intensity of the magnetic field results in a greater change in the apparent weight of the solid phase. As the magnetic field intensity increases, the weaker association of non-specifically bound magnetically-labeled reagent with the well bottom will be overcome, thereby separating it from the specifically bound magnetically-labeled reagent.

The method illustrated in FIG. **5***a* and FIG. **5***b* can also be used to determine the strength of the association between binding members and non-specifically bound magnetically labeled reagent. For example, as the magnet is moved closer to the well, the increasing intensity of the magnetic attractive force at the well bottom begins to pull specifically bound magnetically-labeled reagent from the well bottom to the air-liquid interface where it makes a greater contribution to the aggregate upward force on the well.

It is to be understood that since the present invention involves the assessment of the force or movement manifested by the magnetically-labeled reagent, the various detection methods and reagents described herein are readily adaptable to an automated operation or system.

However, such automated operation or system is not meant to exclude the possibility that some assay operations in an automated system may be carried out manually.

The present invention will now be illustrated, but is not intended to be limited, by the following examples.

EXAMPLES

Example 1

Magnetically Assisted Magnetic Label Measurement

A plastic support was inserted into the pan receptacle of a electronic balance (Mettier AE 163; Mettier Instrument Corporation, Heightson, N.J.) in a manner illustrated in FIG. 1. The support included a hole in the top cross piece to accommodate a single microtiter well (Nunc snap-apart, eight well module strips; Nunc Incorporated, Naperville, Ill.) so that the bottom of the well would extend below the bottom of the cross piece. A neodymium-iron-boron fixed magnet (Racoma Incorporated, Boonton, N.J.) was placed on the end of a plastic bar attached to the mechanical stage of a microscope so that movement of the mechanical stage allowed the accurate and reproducible positioning of the magnet below the bottom of the suspended microtiter well.

Superparamagnetic particles (polystyrene/vinyl/-COOH/magnetite particles; Bang's Laboratories, Incorporated, Carmel, Ind.) were supplied as a 10% (w/v) suspension. This suspension was diluted ten-fold with water to a concentration of 10 milligrams of particles per milliliter. Aliquots ranging from 5–40 microliters (50–400 μg of particles) were pipetted into the microtiter well which was then inserted into the support. The particle suspension was further diluted ten-fold to obtain another set of aliquots containing 5–40 micrograms of particles to assess the response from smaller quantities of particles. A measured quantity of magnetic particles was placed in the microtiter well, and the balance was zeroed with the magnet withdrawn from the vicinity of the well.

In order to measure the force generated upon approach of the magnet to the magnetic particles in the well, the magnet was positioned approximately two millimeters (2 mm) below the well bottom and was moved toward the well bottom in a series of small incremental moves. The balance readout was noted after each movement, and the last reading obtained before the magnet touched the well bottom was recorded. Using this procedure, an average enhancement factor of over 700 was observed, and the effect appeared substantially linear down to at least 20 micrograms of magnetic particles as illustrated by the data presented in Table 2.

TABLE 2

Weight of magnetically-labeled reagent in a well (micrograms)	Force on the reagent due to magnetic field (micrograms)	Ratio of Weight/Force
5	4,500	900
11	3,300	300
16	11,000	687
21	13,500	642
27	20,000	740
32	25,000	781
38	25,000	658
43	33,000	767
54	40,000	741
110	97,000	880
160	150,000	937
210	163,000	776
270	130,000	481
320	310,000	968
380	332,000	874
430	275,000	640

Example 2

Magnetically Assisted Avidin-Biotin Binding Assay

The following reagents and samples were used in a binding assay:

A magnetically-labeled reagent was utilized comprising streptavidin-coated paramagnetic microparticles (Advanced Magnetics, Cambridge, Mass.; average one micron in diameter, with 5×10^8 particles per mg, supplied as a 5 mg/ml suspension). The binding capacity of the particles was 3.2 micrograms of biotin per milliliter of suspension. Test samples contained various concentrations of biotin in a phosphate buffered saline solution.

A solid phase was utilized comprising snap-apart polystyrene microtitre wells (Nunc 8-well microwell module strips; Nunc Incorporated, Naperville, Ill.) which had been coated with biotinylated bovine serum albumin (biotin-BSA 8.9 moles of biotin per mole of BSA; Sigma Chemical Company, St. Louis, Mo.;). The biotin-BSA was dissolved in phosphate buffered saline (PBS), pH 7.2 to a concentration of 50 micrograms/milliliter, and aliquots (100 μl) were pipetted into each well. Following incubation for one hour at 37° C., the solution was removed from the wells and replaced with 400 microliters of 1% BSA (unbiotinylated) in PBS as an overcoat. Incubation was continued at 37° C. for an additional 45 minutes. The wells were then emptied and washed with PBS using a wash bottle. The overall result of this procedure was to immobilize biotin molecules to the bottom of the microtiter wells (as biotin-BSA) and to inactivate the wells to further non-specific binding of protein by overcoating with unbiotinylated BSA.

The magnetically-labeled reagent was first combined with the test sample, thereby forming a reaction mixture which was incubated for one hour at 37° C. An aliquot (80 μl) of each reaction mixture was transferred to the solid phase, where it was further incubated for one hour at 37° C. to effect avidin-biotin binding. The unbound magnetically-labeled reagent was removed from the reaction mixture by means of magnetic attraction.

Once combined, the free biotin from the test sample proceeded to bind to the available biotin-binding sites on the avidin moieties of the avidin-coated magnetic particles, thereby inhibiting the subsequent capture of the magnetically-labeled reagent by the immobilized biotin on the well bottom. The degree of inhibition depended on the concentration of the free biotin in the test sample. Thus, the amount of magnetically-labeled reagent that was bound by the solid phase was inversely proportional to the amount of biotin in the test sample.

The magnetic responsiveness of the magnetically-labeled reagent bound to the bottom of each well was determined using an apparatus substantially as described in Example 1, above. The weight change due to the magnetic responsiveness of the immobilized mag-

netically-labeled reagent in each well was recorded as a function of the quantity of free biotin from the test sample present during incubation.

FIG. 6 illustrates the assay results. The balance means detected a decreasing force change, from 12 milligrams to zero milligrams, as the free biotin concentration in the test sample was increased from 0 nanograms/milliliter to 125 nanograms/milliliter (80 μl assayed). Thus, as the amount of free biotin was increased in a test sample, the amount of magnetically-labeled reagent which bound to the solid phase proportionately decreased, and there was a corresponding decrease in the apparent weight change of that bound reagent upon the application of a magnetic field.

FIG. 7 illustrates the assay results plotted as a percent inhibition of the magnetically enhanced weight of the captured magnetically-labeled reagent resulting from the presence of free biotin in the test sample. Fifty percent (50%) inhibition was observed at a free biotin concentration of 40 nanograms/milliliter. From these results, it was determined that the assay configuration provided an assay for free biotin in the test sample with a sensitivity in the femtomolar range.

Example 3

Balance Mean and Magnetically Assisted Measurements

To further explore the potential of the magnetically assisted magnetic label assay concept, a Cahn Model D-200 electronic microbalance (Cahn Instruments Incorporated, Cerritos, Calif.) was used. This balance consists of a balance beam connected to the rotor of an electric motor. A movement of the beam, as the result of the presence of a weight in one of the hanging pans, is sensed by an optical positioning device, and a current is sent to the motor sufficient to return the beam to its original position. The magnitude of this current is converted by the electronic circuitry of the balance into a weight readout.

An apparatus for precisely positioning a fixed magnet was designed and assembled from three precision positioning tables (Daedal Division of Parker Hannifin Corporation, Harrison City, Pa.). Two of the tables were micrometer adjusted and used to position the magnet in the horizontal X and Y directions. The third table, mounted vertically, was driven by a microstepper motor to control the movement of the magnet in the vertical, or Z direction (Compumoter Division of Parker Hannifin Corporation, Rohnert Park, Calif.). A fixed magnet (Racoma 35; Recoma, Inc., Boonton, N.J.) was attached to the Z table by a bracket which positioned the magnet inside an enclosure that surrounded the balance pans to shield the pans from air currents. The motor movement was controlled by microprocessor circuitry interfaced with a computer. Acceleration, velocity and distance of movement, as well as final position were programmed into the computer such that complex repetitive movements could be executed automatically. A movement of one inch comprised 100,000 microsteps, and controlled movements of one microstep were possible.

The relationship of the magnetic enhancement factor to the quantity of magnetically-labeled reagent was confirmed using the Cahn balance. A ten microliter aliquot of a suspension containing paramagnetic particles as described in Example 1(100 μg/ml) was pipetted onto the balance pan, the balance was equilibrated, the magnet was brought into proximity of the pan and the change in readout was noted. Additional ten microliter aliquots were then added, and the process was repeated until a total of 100 microliters had been added. Table 3 illustrates the relationship between the quantity of magnetically-labeled reagent in the well and the force exerted upon the reagent (measured as an increase in weight) by the magnetic field. As the quantity of magnetic reagent increased, the balance deviation due to the magnet movement increased linearly.

TABLE 3

Quantity of paramagnetic microparticles in well (micrograms)	Balance Readout (mg) magnet away	magnet close	Difference	Ratio
1	8.05	8.74	0.69	690
2	17.68	18.70	1.02	510
3	27.19	28.65	1.46	487
4	36.78	38.91	2.13	533
5	46.18	49.09	2.91	582
6	55.06	58.80	3.74	623
7	63.80	68.29	4.49	641
8	72.39	77.70	5.31	664
9	80.63	86.86	6.23	692
10	88.76	95.88	7.12	712

Example 4

Magnetically Assisted Antibody Assay A magnetically assisted inhibition immunoassay was demonstrated using the following reagents:

(a) A magnetically-labeled reagent was utilized comprising paramagnetic particles (Advanced Magnetics; Cambridge, Mass.) coated with antibody directed against mouse IgG (heavy and light chain) as a one milligram/milliliter suspension (5×10^8 particles/ml).

(b) A solid phase was used comprising the wells of a microtitre plate coated with mouse IgG (100 microliters of a 50 μg/ml solution, in a 1% carbonate buffer, pH 8.6). The solid phase was then overcoated with 1% BSA in PBS.

(c) Test samples (80 microliters) contained various concentrations of free mouse IgG in a PBS buffered solution.

The magnetically-labeled reagent was incubated with the antibody immobilized on the solid phase in the absence of free mouse IgG. The magnetically-labeled reagent bound to the solid phase and resisted removal upon application of a magnetic field. The binding of the magnetically-labeled reagent was shown to be specific for the immobilized mouse antibody, because the same magnetically-labeled reagent was removed by the application of the same magnetic field when incubated with a solid phase which had been coated with BSA alone.

When the magnetically-labeled reagent was first incubated with the free mouse antibody, the subsequent binding of the magnetically-labeled anti-mouse IgG to the mouse IgG-coated on the well bottom was inhibited. The quantity of magnetically-labeled reagent remaining bound to the solid phase, after the magnetic separation of unbound magnetically-labeled reagent, was measured by placing the well on a balance pan, zeroing the balance, and then moving the magnet into position.

FIG. 8 illustrates the effect of repeatedly moving the magnet first towards and then away from the proximity of the bottom of the well in which there had been no

free mouse IgG during incubation, i.e., no inhibition of magnetically-labeled reagent binding to the immobilized antibody in the well bottom; wherein line (a) in FIG. 8 shows the magnetic enhancement of weight upon the approach of the magnetic field and line (b) in FIG. 8 shows the return to the zero point upon the removal of the magnetic field. FIG. 9 depicts the record of a single cycle of the application and withdrawal of the magnetic field under these conditions, which produced an apparent weight change of 5.8 milligrams.

The presence of free mouse IgG (at a concentration of 2.5 μg/ml), during the incubation, caused a decrease in the observed effect of the magnetic field. As shown in FIG. 10, incubation of the magnetically-labeled reagent with free mouse IgG at a concentration of 2.5 micrograms/milliliter resulted in a change of apparent weight of only to 2.8 milligrams (note the change in vertical axis units from FIG. 9). The effect of the presence of various concentrations of free mouse IgG during the incubation was determined as percent inhibition of the value obtained in the absence of free mouse IgG.

FIG. 11 illustrates the results which were plotted as a function of free mouse IgG concentration versus per cent inhibition of weight change. The data illustrate a classical inhibition curve with 50% inhibition resulting from the presence of free mouse IgG at a concentration of one microgram/milliliter.

Example 5

Magnetically Assisted Binding Affinity Measurements

The following experiment was performed to measure the binding affinity between a given pair of binding members. The method involved the use of microtiter wells, which had been cut to provide a reduced well wall height of approximately five millimeters, and a magnetic means which approached the wells from above.

The strength of the association between the captured magnetically-labeled reagent and the solid phase was measured. Mouse IgG was immobilized in the well, the well was overcoated with a 1% BSA solution, a suspension of anti-mouse IgG antibody coated magnetic particles was placed in the well, and the reaction mixture was incubated at 37° C. for 1 hour to allow binding to take place.

A magnetic field was moved in discrete steps into proximity with the top of the well, thereby causing a controlled series of increases in the upward attractive force exerted on the magnetically-labeled antibody in the well. FIG. 12 illustrates the procedure, wherein FIG. 12(*a*) illustrates the magnetically-labeled reagent, some of which is immobilized on the solid phase as a result of a binding reaction, and the balance readout prior to the approach of a magnet to the solid phase. The initial movement (50,000 microsteps) of the magnet toward the surface of the suspension caused that magnetically-labeled antibody which was not bound to the immobilized antibody to migrate to the air-liquid interface of the suspension. As the magnet was moved closer to the surface (in 5,000 microstep movements) there was a corresponding increase in the attractive force upon the free particles resulting in an observable decrease in the weight of the vessel with each discrete movement, as shown in FIG. 12(*b*). The free particles collected at the interface exerted an upward force against the surface tension of the liquid surface, thereby causing an observable decrease in the apparent weight of the well. The decrease in weight was determined by a balance means substantially in accordance with the method described in Example 1, above. The magnetically-labeled antibody which was bound to the immobilized antibody on the well bottom also exerted an upward force in the magnetic field. The force exerted by the bound reagent, however, was much less than that exerted by the free reagent at the surface due to the greater distance of the bound reagent from the magnet. As the magnet approached the top of the well, thereby causing the upward force on the magnetically-labeled antibody to increase, the magnetically-labeled antibody that was bound to the immobilized binding member on the well bottom began to dissociate from the well bottom and migrate to the liquid surface, as shown in FIG. 12(*c*).

FIG. 13 illustrates the measurement of the association force of the magnetically-labeled reagent and the solid phase. During the initial 50,000 microstep movement [FIG. 13(*a*)] of the magnet toward the top of the well, the attractive magnetic field was relatively weak, and the decreases in apparent weight resulted from the increased upwards force exerted upon the free magnetically-labeled antibody which was collected at the air-liquid surface. As the magnet was moved closer to the surface (in 5,000 microstep movements) there was a corresponding increase in the attractive force upon the free particles resulting in an observable decrease in the weight of the vessel with each discrete movement of the magnet [FIG. 13(*b*)]. Initially, there was no observed weight change when the magnet was stopped between movements, indicating that no change in particle position was occurring between the changes in magnetic field intensity.

As the magnet approached the top of the well, and the force on the bound particles increased, the magnetically-labeled antibody that was bound to the immobilized binding member on the well bottom began to dissociate allowing the magnetically labeled reagent to migrate to the liquid surface. As the dissociated particles reached the liquid surface, they were in a region of a greater attractive magnetic force, and therefore, these particles exerted a greater upward force on the well. This forced dissociation of the magnetically-labeled antibody from the well bottom, and the subsequent migration to the liquid surface, was manifested as a gradual decrease in well weight between movements of the magnet. The change in apparent weight was seen as a deviation of the weight trace from the horizontal between movements [FIG. 13(*e*)] of the magnet. As the magnet was withdrawn in a series of discrete movements [FIG. 13(*c*)] from the proximity of the well, the apparent weight changes between movements reverted to zero [FIG. 13(*f*)].

When the magnet was again advanced toward the same well 13(*d*), there was little apparent weight change between the movements of the magnet, demonstrating that all of the magnetically-labeled reagent which would dissociate from the well bottom under a given level of magnetic force had already dissociated during the first approach of the magnetic field [FIG. 13(*g*)]. Thus, any further changes in apparent weight were mostly due to changes in the magnetic field force on the dissociated magnetic microparticles as the magnet moved, with little contribution due to the further disassociation of magnetically-labeled reagent from the well bottom.

Since it is the binding affinity between the magnetically-labeled reagent and the solid-phase reagent which determines its ability to resist dissociation by the applied magnetic field force, the results demonstrated that the association constants between binding members can be quantitatively determined by means of magnetically assisted magnetically-labeled reagent measurements. The attractive magnetic field intensity required to overcome the association of the binding members is a direct measure of the association constant between the binding members.

Example 6

Magnetically Assisted Binding Measurements of Unbound Reagent

A BSA-coated vessel was incubated with a suspension of anti-mouse IgG-coated magnetic particles at 37° C. for 1 hour. The magnetic response measurements (performed substantially in accordance with the method describe in Example 5, above) revealed a decrease of three milligrams in weight when the magnetic means approached the top of the vessel. A vessel containing the same quantity of anti-mouse IgG-coated magnetic particles, but which also contained immobilized mouse IgG (overcoated with BSA), exhibited less than a one milligram weight change, thereby indicating that enough magnetically-labeled reagent had been captured by the immobilized antibody on the well bottom to decrease by two-thirds the magnetic response measurement due to the unbound magnetic particles which had migrated to the liquid surface.

The addition of free mouse IgG, during the incubation of the magnetically-labeled reagent and the solid phase, was found to inhibit the binding of the magnetically-labeled reagent to the immobilized mouse IgG on the well bottom, thereby allowing the unbound magnetically-labeled reagent to migrate to the liquid surface under the influence of the magnetic field and display a greater magnetic responsiveness. In this way, inhibition immunoassays similar to that shown in FIG. **9–11** could be monitored by apparent weight change caused by magnetic particle levitation, i.e., detection of unbound magnetically-labeled reagent. Because magnetic attraction falls off rapidly with distance (as previously shown in FIG. **13**), the movement of the free magnetically-labeled reagent nearer to the magnet greatly enhances its influence relative to that of the bound magnetically-labeled reagent. This permits a determination of the relative degree of magnetically-labeled reagent binding without requiring the removal of the free magnetically-labeled reagent from the well.

Example 7

Magnetically Assisted Binding Measurements in a Two Particle Assay

An alternative assay method can involve the use of a particulate solid phase. The magnetically-labeled reagent comprises a binding member conjugated to a magnetic label having an average diameter of 0.05 microns or less. The magnetically-labeled reagent is mixed with an amount of larger nonmagnetic particles (e.g., polystyrene microparticles, diameter of about 5.0 microns) to form a reaction mixture. An immobilized binding pair member on the surface of the nonmagnetic particles causes the magnetically-labeled reagent to bind to the larger particles in the presence of the analyte.

Following a binding reaction, the application of a magnetic field causes the unbound magnetically-labeled reagent to rapidly migrate toward the magnetic means. The magnetically-labeled reagent that is bound to the more massive nonmagnetic particles migrates at a much slower rate in the magnetic field, thereby providing for the discrimination between bound and free magnetically-labeled reagent. After the separation of the unbound magnetically-labeled reagent, that magnetically-labeled reagent that is bound to the non-paramagnetic particles is subjected to analysis using the magnetically assisted magnetically-labeled measurement methods described above.

Example 8

Magnetically Assisted Binding Assay Using a Suspended Field Generator

According to this example an assay was performed with a magnet attached to the balance means. As shown in FIG. **14**, a magnet **60** was suspended from the balance beam **65**, of a Cahn Model D-200 electronic microbalance (Cahn Instruments Inc.; Cerritos, Calif.). The sample vessel **20** was placed on a pedestle **30** which was attached to a computer controlled elevator device **80**. A cylindrical telescoping housing **85** was also attached to the balance and the elevator to shield air currents. When the sample vessel containing a magnetically attractable label **10** was positioned in the proximity of the magnet, a force was exerted on the magnet as it was attracted to the magnetically attractable label in the vessel. As a result, the apparent weight of the magnet increased and the increase was measured by the balance means. With the magnet attached to the balance, the balance is not disturbed during the changing of the sample vessels and the weight of the magnet is constant when the sample vessel is not in the magnet's proximity. Hence, the balance did not have to be rezeroed between each reading step.

An assay for human thyroid stimulating hormone (TSH) was performed using this arrangement. Antibody which was specific for the beta chain of TSH was dissolved at a concentration of 20 ug/ml in phosphate buffered saline (PBS). Aliquots (100 ul) of this solution were pipetted into Nunc snap-apart microtiter wells (Nunc Inc.; Naperville, Ill.) and then incubated at 37° C. for 1 hr. The well contents were then removed and the wells were overcoated with 300 ul of a 1% solution of bovine serum albumin (BSA)in PBS by incubation at 37° C. for 45 minutes. The BSA solution was removed from the wells and replaced with 200 ul aliquots of the TSH containing standards from the Abbott IMx® Ultrasensitive hTSH kit (Abbott Laboratories; Abbott Park, Ill.) before being incubated at 37° C. for 1 hour. After this incubation, the well contents were removed, the wells were washed with PBS, and 300 ul of a 0.002% suspension of superparamagnetic microparticles (0.8 um in diameter, Bang's Labs, Indianapolis, Ind.) which had been coated with an antibody specific for the alpha subunit of TSH were added to each well. The contents of the well were incubated at 37° C. for 1 hour. During this incubation, the particles settled to the bottom of the well and became bound to the immobilized TSH antigen also located at the bottom of the well. After the incubation, each well was filled above its rim with PBS and a glass cover slip was placed over the top of the well such that no air bubbles were trapped beneath it.

The covered well was then placed on the elevator and brought into the proximity of a ring-shaped magnet which served to attract the particles in the well and cause the unbound particles to move up to the underside of the cover slip. The cover slip was removed while in the proximity of the magnet which caused the unbound particles to be removed with it. The well was then centered above the corner of a rectangular magnet, which served to overcome the association between the bound particles and the well bottom. The result was the accumulation of the particles in a dot in the center of the well bottom. All of the liquid was removed from the well and the well was allowed to dry. The well was then inverted and placed on an elevator below the Cahn balance (as described above) and elevated into proximity of the magnet suspended from the balance beam. Weight deflections caused by the presence of the dried dot of microparticles were recorded and plotted as a function of the amount of TSH antigen contained in the standard which had been incubated in each well.

The data from the experiment is shown below in Table 4 which is also plotted and shown in FIG. **15**. The data (shown in Table 4) for each TSH calibrator is an average taken from eight wells.

TABLE 4

Calibrator	TSH Concentration	Weight Change
A	0.0 ul.U/ml	1.25 mg
B	0.5 ul.U/ml	1.45 mg
C	2.0 ul.U/ml	2.6 mg
D	10.0 ul.U/ml	3.6 mg
E	40.0 ul.U/ml	3.9 mg
F	100.0 ul.U/ml	4.1 mg

Example 9

Magnetically Assisted Binding Assay Using a Field Generator Attached to a Measurement Means

In an alternative assay configuration, shown in FIG. **16**, a magnet **60** is placed on the pan **40** of a top-loading microbalance **50** to form a magnet-balance configuration. Using support means **30** the bottom of a reaction vessel **20** which contains magnetically attractable material **10** is then positioned in the proximity of the magnet. Apprarent weight changes are then noted. The use of this configuration to analyze the results of an assay described in Example 8 has several advantages. For example, after removal of the unbound magnetically-labeled reagent (as described in Example 8), the well and its contents can be placed directly on the support and the apparent weight change of the magnet noted without removing the well contents or inverting the well.

Example 10

Performance of Magnetically Assisted Binding Assays Without Removing Unbound Reagent From the Reaction Vessel

The magnet-balance configuration described in Example 9 allows the performance of assays where the unbound particles are not separated from the reaction vessel containing the bound particles. As illustrated in FIG. **17***a*, a conical magnet **60** is placed on the pan **40** of a top-loading Merrier UMT-2 microbalance (Mettier Instrument Corporation, Heightson, N.J.) **50** to form a magnet-balance configuration. Using support means **30** the bottom of a reaction vessel **20** which contains magnetically attractable material **10** was then positioned in the proximity of the magnet.

Using such an arrangement, an assay for TSH was performed using wells prepared with the A, B, C, D and F TSH containing standards from the Abbott IMx ® Ultrasensitive hTSH kit (Abbott Laboratories; Abbott Park, Ill.) as described above in Example 8. After the microparticle incubation step (as described in Example 8) the wells were placed in a support **30** which suspended the well over the center of a conical shaped magnet sitting on the pan of a Mettler UMT-2 microbalance (Mettier Instrument Corporation, Heightson, N.J.). As shown in FIG. **17***b*, when the magnetic field was applied by the magnet **60**, the non specifically bound magnetically-labeled reagent migrated to a postion in the center of the well **20** above the point of the magnet **60**. Because of the shape of the magnetic field generated by the conical magnet **60**, particles which migrated to the center of the well exert a much stronger attractive force on the magnet than do the specifically bound magnetically-labeled reagent which has resisted migration. As a result the unbound particles contribute much more to the observed weight change as determined by the balance. The apparent magnet weight changes as a function of time for the above assay are shown in FIG. **18**. As shown in FIG. **18**, the weight change observed was related to the amount of TSH antigen contained in the standard and thereby contained in the well. Clearly, increasing quantities of antigen increase the binding affinity of the magnetically-labeled reagent for the bottom of the well. For example, the magnetically labeled reagent incubated with the A calibrator (which has no TSH antigen) showed the weakest binding to the well bottom, and therefore, showed the greatest apparent weight change.

It will be appreciated by one skilled in the art that many of the concepts of the present invention are equally applicable to other analytes, binding pair members, assay formats and magnetically-attractable materials. The specific embodiments described are intended as examples rather than as limitations. Thus, the description of the invention is not intended to limit the invention to the particular embodiments disclosed, but it is intended to encompass all equivalents and subject matter within the scope of the invention as described above and as set forth in the following claims.

Example 11

Magnetically Assisted Binding Assay for Alpha Fetal Protein

The solid phase (Nunc microtiter wells available from Nunc Inc.; Naperville, Ill., as used in the examples above) was coated with anti-AFP antibody, overcoated with 1% BSA, and incubated with standard amounts of Alapha Fetal Protein (AFP) calibrators from an Abbott AFP IMx ® diagnostic kit (Abbott Laboratories; Abbott Park, Ill.). As illustrated in FIG. **19***a* and FIG. **19***b*, the reaction vessel **20** was washed and incubated with a magnetically-labeled anti-AFP antibody reagent **10**. Following incubation, a microscope cover slip **15** was placed on top of the well such that the liquid contents of the well contacted the underside of the cover slip thereby leaving no air space. The unbound magnetically-labeled reagent in the well was separated from the bound magnetically-labeled reagent by elevating the reaction vessel in the proximity of a magnet **60** affixed to the beam of a Cahn microbalance **50** (Cahn Instru-

ments Incorporated, Cerritos, Calif.). The unbound magnetically-labeled reagent was pulled to the underside of the cover slip lid of the reaction vessel. In this position, the unbound particles collected on the underside of the cover slip. Because the unbound particles were closest to the magnet, they attracted the magnet most strongly, causing its apparent weight to increase as measured by the balance. This procedure was repeated and the weight change was recorded for each calibrator. The results of the experiment are shown in Table 5.

The results shown in Table 5 show that the higher the concentration of AFP antigen in the calibrator solution, and therefore in the reaction vessel, the smaller the apparent increase in the weight of the magnet. This was due to the magnetically-labeled reagent binding to the AFP antigen bound to the solid phase reagent thereby leaving less unbound magnetically-labeled reagent to migrate to the cover slip and cause the apparent weight change. The magnitude of the apparent weight change of the magnet was inversely proportional to the amount of AFP calibrator in the reaction vessel.

TABLE 5

AFP Calibrator	AFP (ng/ml)	Balance weight
A	0	5.8
B	15.0	4.3
C	50.0	2.6
D	100.0	1.2
E	200.0	0.4
F	350.0	−0.1

As shown in FIG. 20, the rates of the of the apparent weight change of the magnet during an assay performed in this way can also serve as a measure of the degree of association of the bound magnetically labeled reagent for the solid phase. The rates of change of the apparent weight of the magnet with time are plotted. This approach avoids the necessity of removing the unbound particles from the reaction vessel before the read step, allowing the separation and read step to be performed simultaneously.

Example 12

Measurement of the Rate Of Weight Change

The rate of apparent weight change of the magnet during an experiment as described in Example 11 can be controlled by adjusting the distance of the well from the magnet. Movements of the well toward the magnet do not need to be abrupt, but can also be continuous.

What is claimed is:

1. A method for determining the presence or amount of analyte in a test sample, the method comprising the steps of:

(a) contacting said test sample with

(i) a solid-phase reagent comprising a first binding member immobilized on a solid phase, wherein said first binding member specifically binds said analyte, and

(ii) a magnetically-labelled reagent, comprising a second binding member attached to a magnetically-attractable label, wherein said second binding member

specifically binds said analyte, thereby producing unbound magnetically-labeled reagent and magnetically-labeled reagent bound to said solid-phase reagent in relation to the amount of analyte present in said test sample;

(b) partitioning said unbound magnetically-labeled reagent and said bound magnetically-labeled reagent with a separation means;

(c) applying a magnetic field to either or both of said bound and unbound magnetically-labeled reagents with a magnetic field generator means; and

(d) assessing the effect of said magnetic field on said magnetically-labeled reagent or reagents as a measure of the presence or amount of the analyte in the the sample with a measurement means comprising a balance.

2. A method according to claim 1 wherein said first binding member is indirectly immobilized on said solid phase through an ancillary binding member which is directly immobilized on said solid phase and which specifically binds to said first binding member.

3. A method according to claim 1 wherein said second binding member is indirectly attached to said magnetically-attractable label through an ancillary binding member which is directly attached to said magnetically-attractable label and which specifically binds to said second binding member.

4. A method according to claim 1 wherein said partitioning step (b) is accomplished with the use of a magnetic field generator means which is the same as or different from said magnetic field generator means of step (c).

5. A method for determining the presence or amount of analyte in a test sample, the method comprising the steps of:

(a) contacting said test sample with

(i) a solid-phase reagent comprising a first binding member immobilized on a solid phase, wherein said first binding member specifically binds a second binding member, and

(ii) a magnetically labelled reagent, comprising said second binding member attached to a magnetically-attractable label, wherein said second binding member

specifically binds said analyte and said first binding member, thereby producing unbound magnetically-labeled reagent and magnetically-labeled reagent bound to said solid-phase reagent in relation to the amount of analyte present in said test sample;

(b) partitioning said unbound magnetically-labeled reagent and said bound magnetically-labeled reagent with a separation means;

(c) applying a magnetic field to either or both of said magnetically-labeled reagents with a magnetic field generator means; and

(d) assessing the effect of said magnetic field on the magnetically-labeled reagent as a measure fo the presence or amount of the analyte in the the sample with a measurement means comprising a balance.

6. A method according to claim 5 wherein said first binding member is indirectly immobilized on said solid phase through an ancillary binding member which is directly immobilized on said solid phase and which specifically binds to said first binding member.

7. A method according to claim 5 wherein said second binding member is indirectly attached to said magnetically-attractable label through an ancillary binding member which is directly attached to said magnetically-attractable label and which specifically binds to said second binding member.

8. A method according to claim 5 wherein said partitioning step (b) is accomplished with the use of a magnetic field generator means which is the same as or different from said magnetic field generator means of step (c).

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CO CTION

PATENT NO. : 5,445,970
DATED : August 29, 1995
INVENTOR(S) : Thomas E. Rohr

It is certified that error appears in the above-indentified patent and that said Letters Patent is hereby corrected as shown below:

Column 32, line 52, change "fo" to --for--.

Signed and Sealed this

Twenty-seventh Day of February, 1996

Attest:

BRUCE LEHMAN

Attesting Officer

Commissioner of Patents and Trademarks

US005458852A

United States Patent [19]

Buechler

[11] **Patent Number: 5,458,852**

[45] **Date of Patent: Oct. 17, 1995**

[54] **DIAGNOSTIC DEVICES FOR THE CONTROLLED MOVEMENT OF REAGENTS WITHOUT MEMBRANES**

[75] Inventor: **Kenneth F. Buechler**, San Diego, Calif.

[73] Assignee: **Biosite Diagnostics, Inc.**, San Diego, Calif.

[21] Appl. No.: **887,526**

[22] Filed: **May 21, 1992**

[51] **Int. Cl.**[6] **G01N 21/85**

[52] **U.S. Cl.** **422/58**; 422/56; 422/57; 422/61; 422/73; 422/102

[58] **Field of Search** 422/56, 57, 58, 422/61, 73, 102

[56] **References Cited**

U.S. PATENT DOCUMENTS

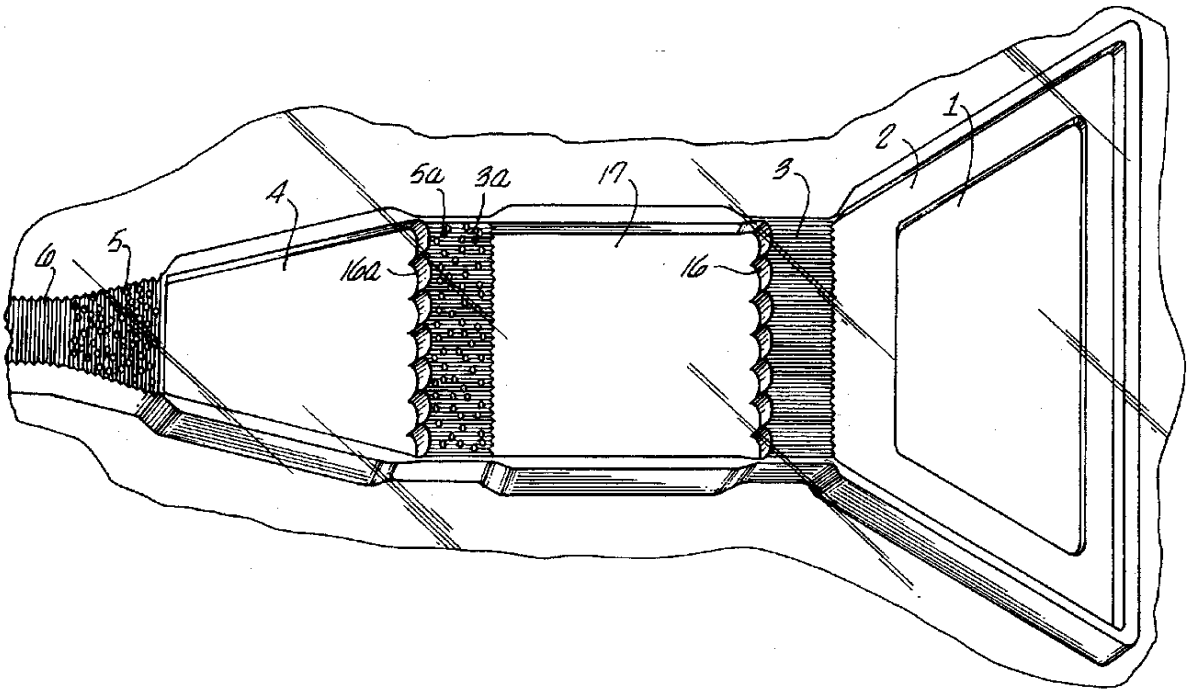
4,426,451	1/1984	Columbus	436/518
4,756,828	7/1988	Litman et al.	435/7
4,757,004	7/1988	Houts et al.	435/7
4,879,215	11/1989	Weng et al.	435/7
4,906,439	3/1990	Grenner	422/56
4,960,691	10/1990	Gordon et al.	435/6
4,963,498	10/1990	Hillman et al.	436/69
5,023,054	6/1991	Sato et al.	422/82.09
5,028,535	7/1991	Buechler et al.	435/7.1
5,051,237	9/1991	Grenner et al.	422/56
5,087,556	2/1992	Ertinghausen	422/56 X
5,089,391	2/1992	Buechler et al.	435/7.1
5,137,808	8/1992	Ullman et al.	422/56 X

Primary Examiner—James C. Housel
Assistant Examiner—Harold Y. Pyon
Attorney, Agent, or Firm—Lyon & Lyon

[57] **ABSTRACT**

The assay devices, assay systems and device components of this invention comprise at least two opposing surfaces disposed a capillary distance apart, at least one of which is capable of immobilizing at least one target ligand or a conjugate in an amount related to the presence or amount of target ligand in the sample from a fluid sample in a zone for controlled fluid movement to, through or away the zone. The inventive device components may be incorporated into conventional assay devices with membranes or may be used in the inventive membrane-less devices herein described and claimed. These components include flow control elements, measurement elements, time gates, elements for the elimination of pipetting steps, and generally, elements for the controlled flow, timing, delivery, incubation, separation, washing and other steps of the assay process.

15 Claims, 8 Drawing Sheets



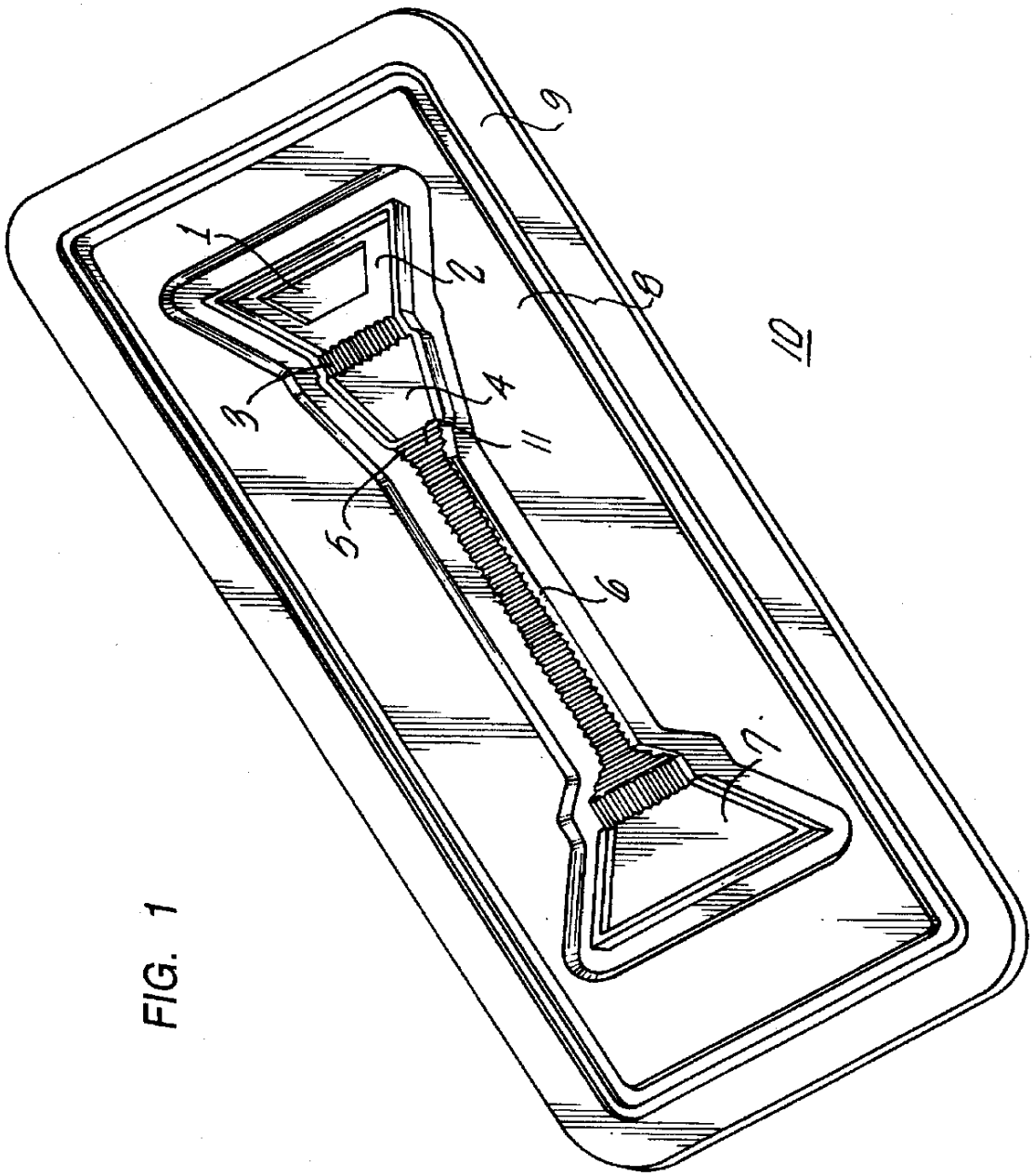


FIG. 1

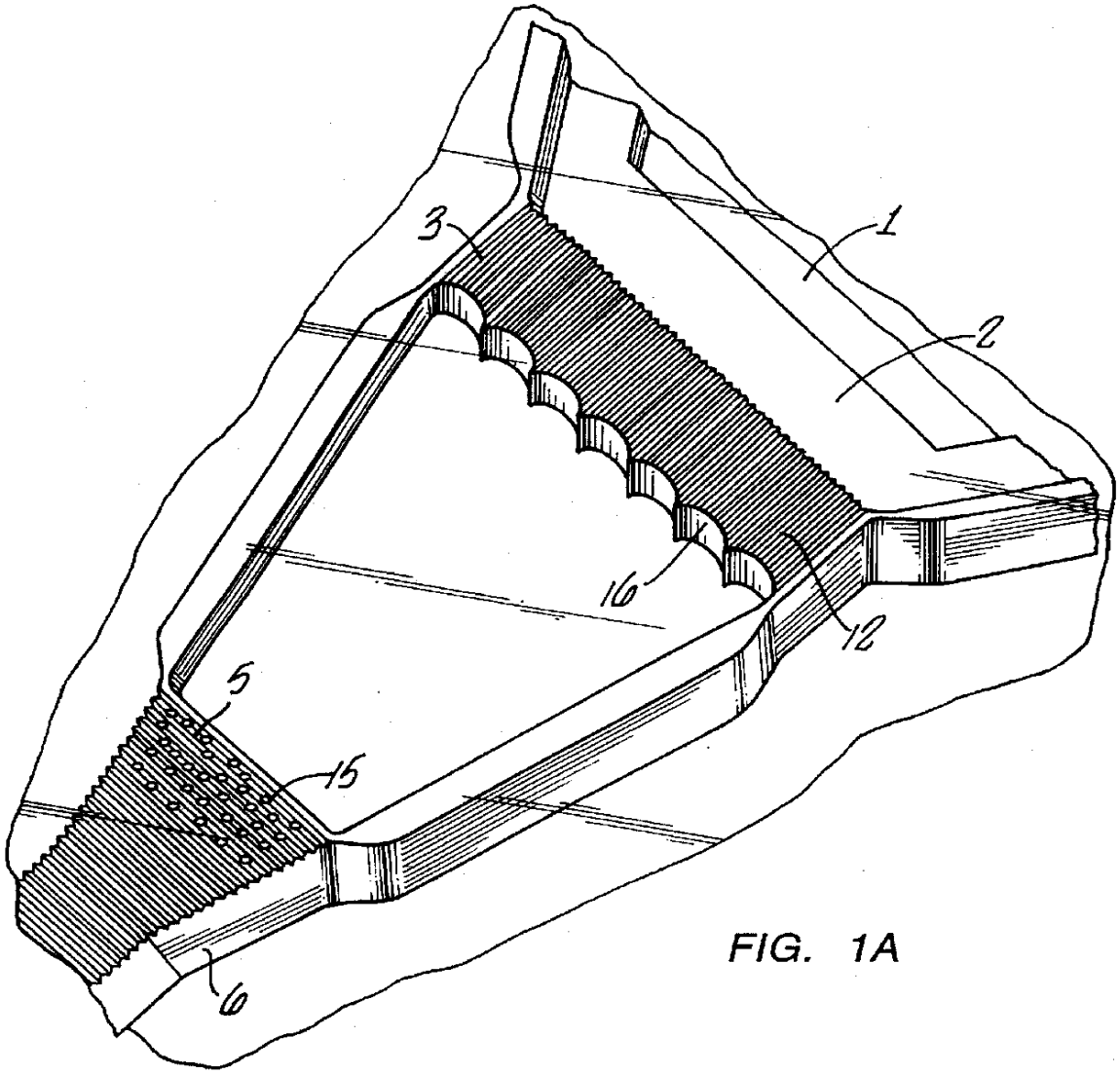


FIG. 1A

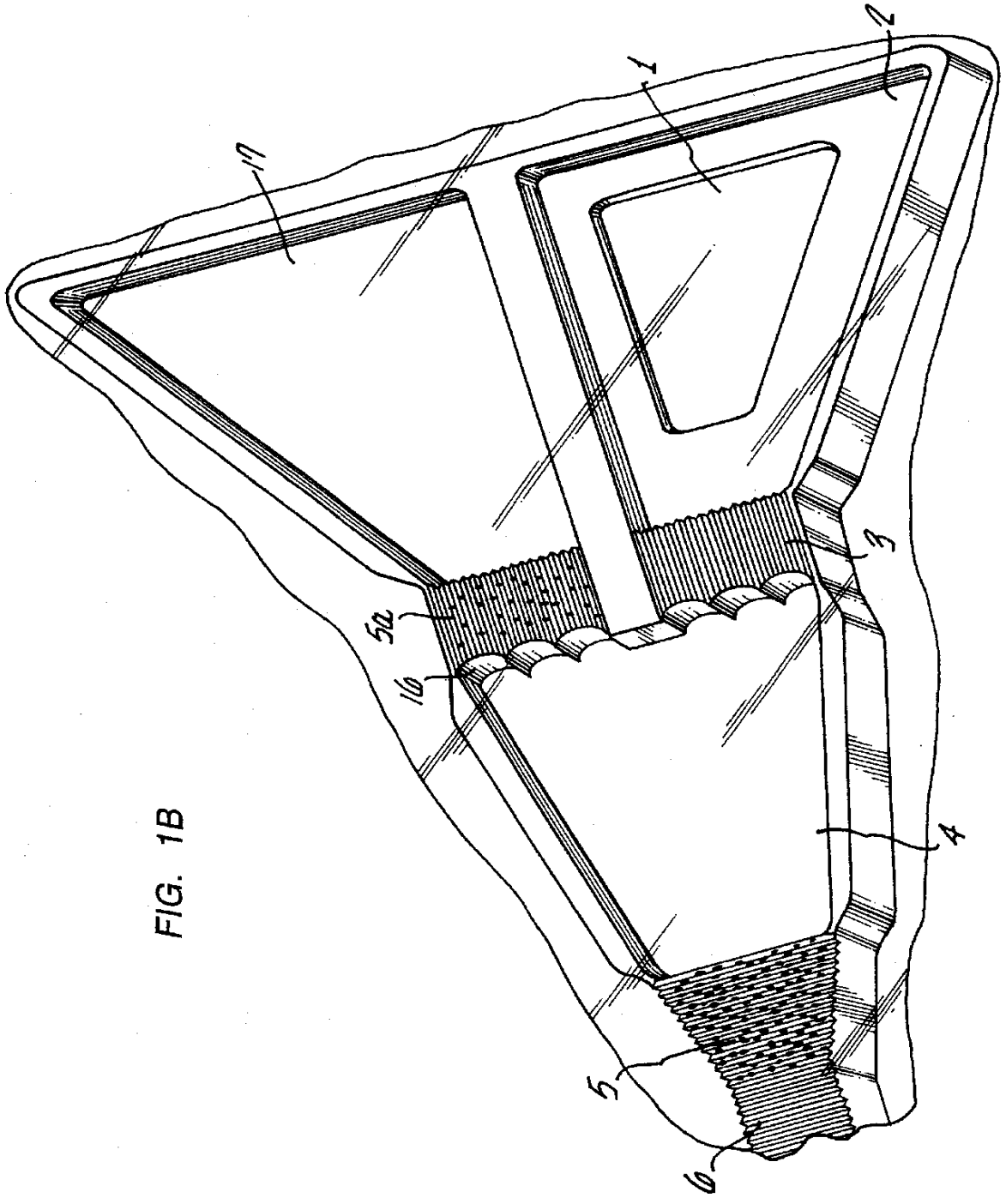


FIG. 1B

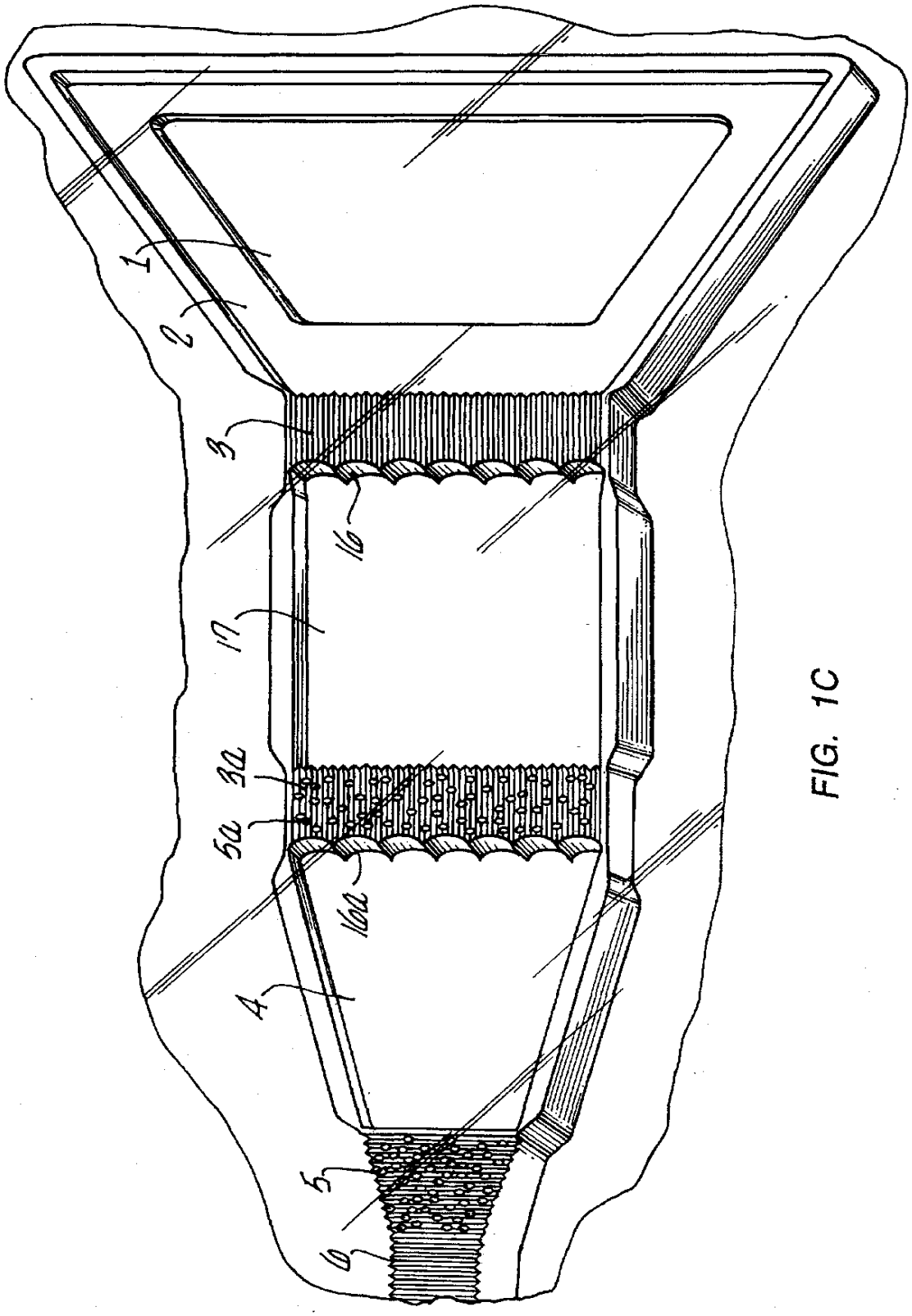


FIG. 1C

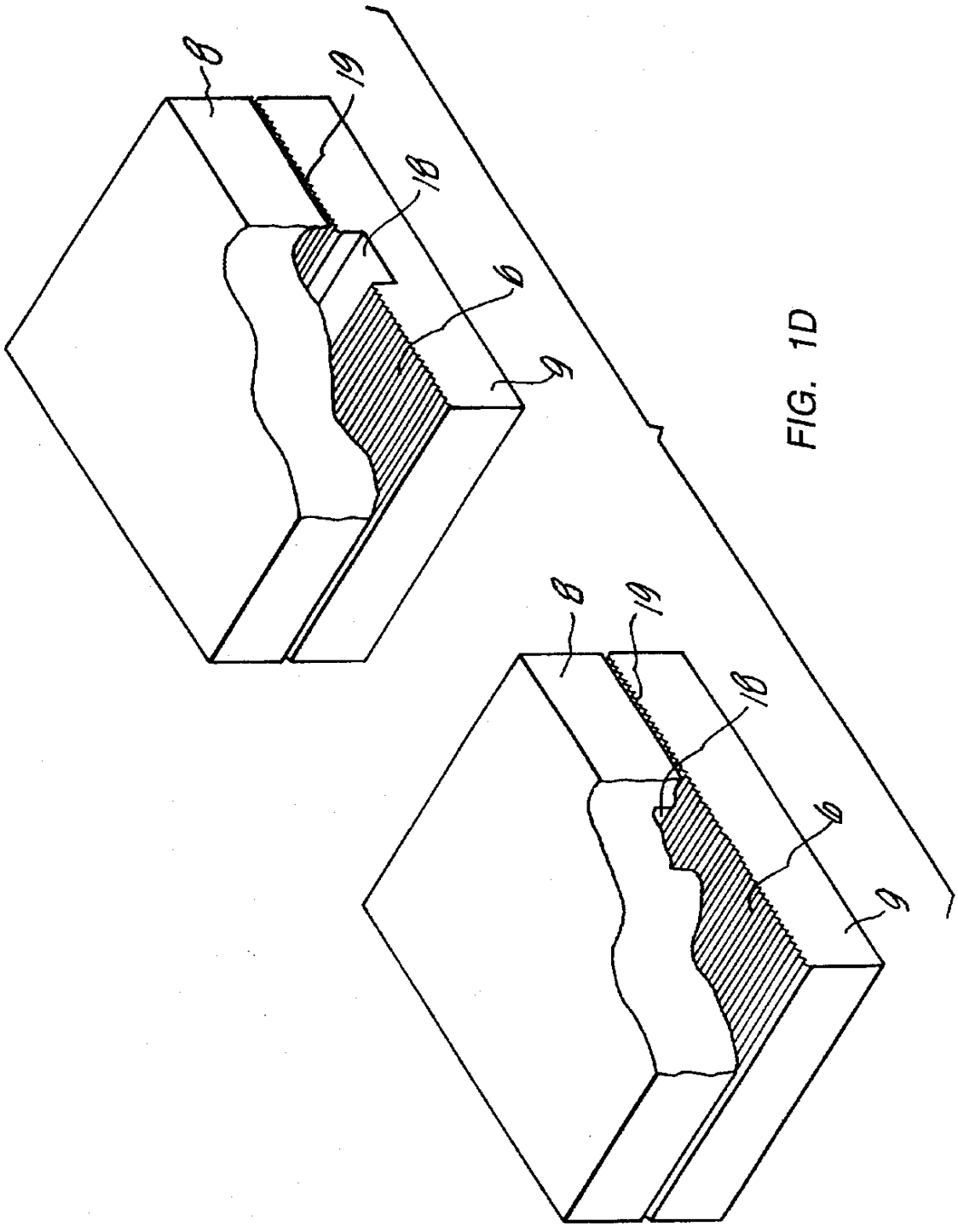


FIG. 1D

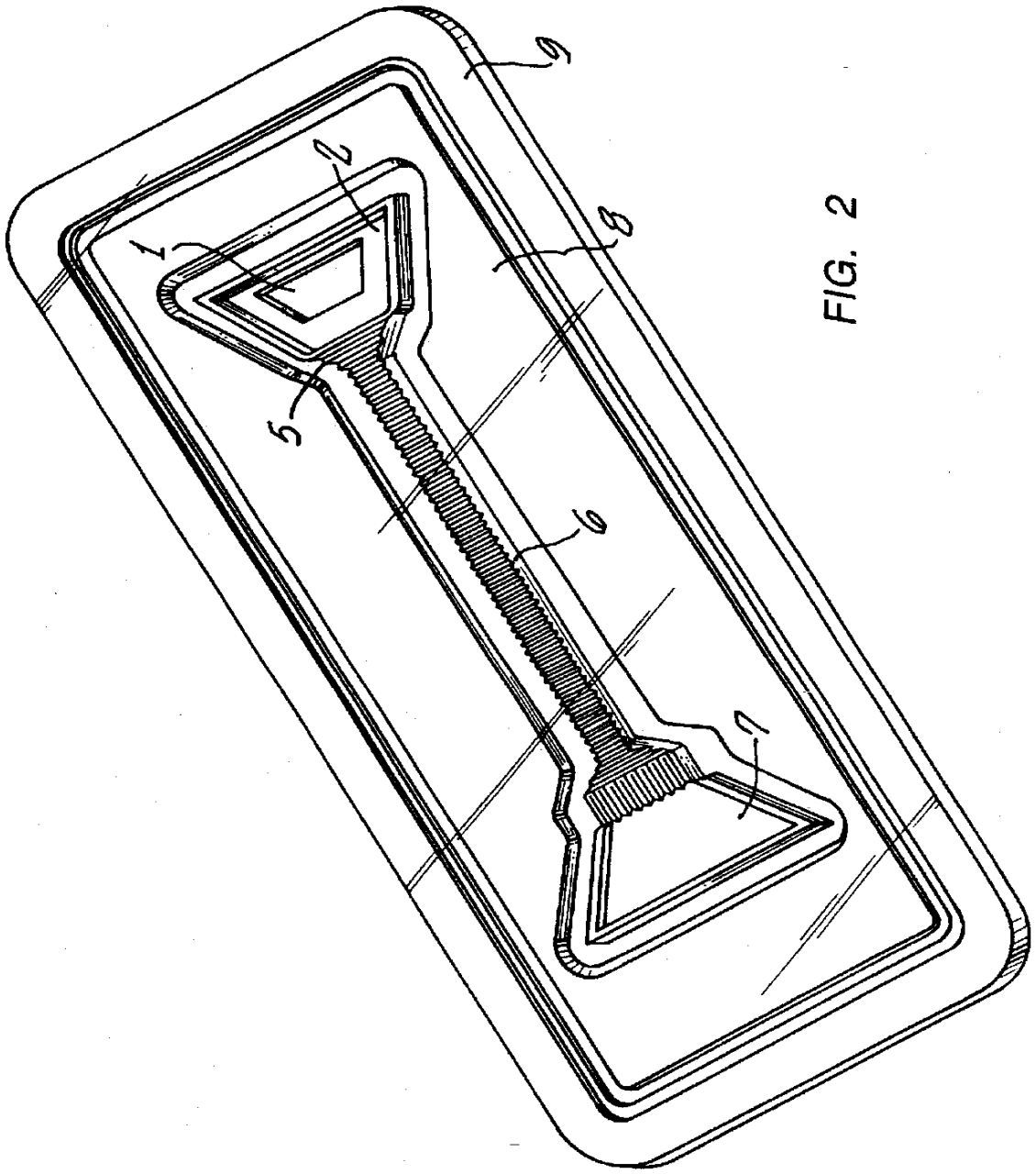


FIG. 2

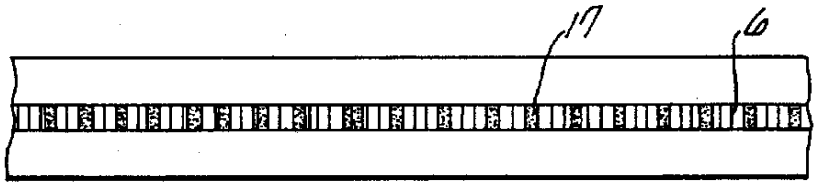


FIG. 3A

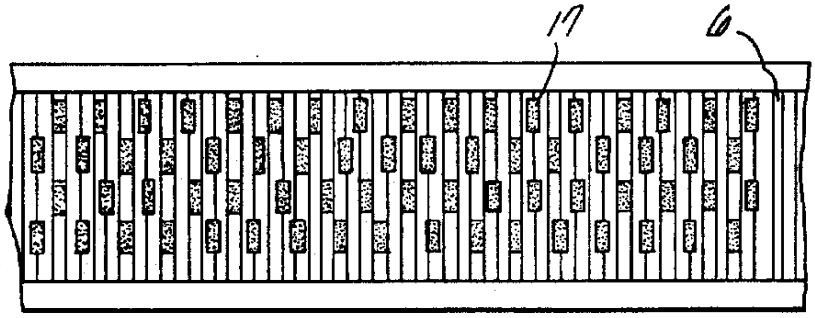


FIG. 3B

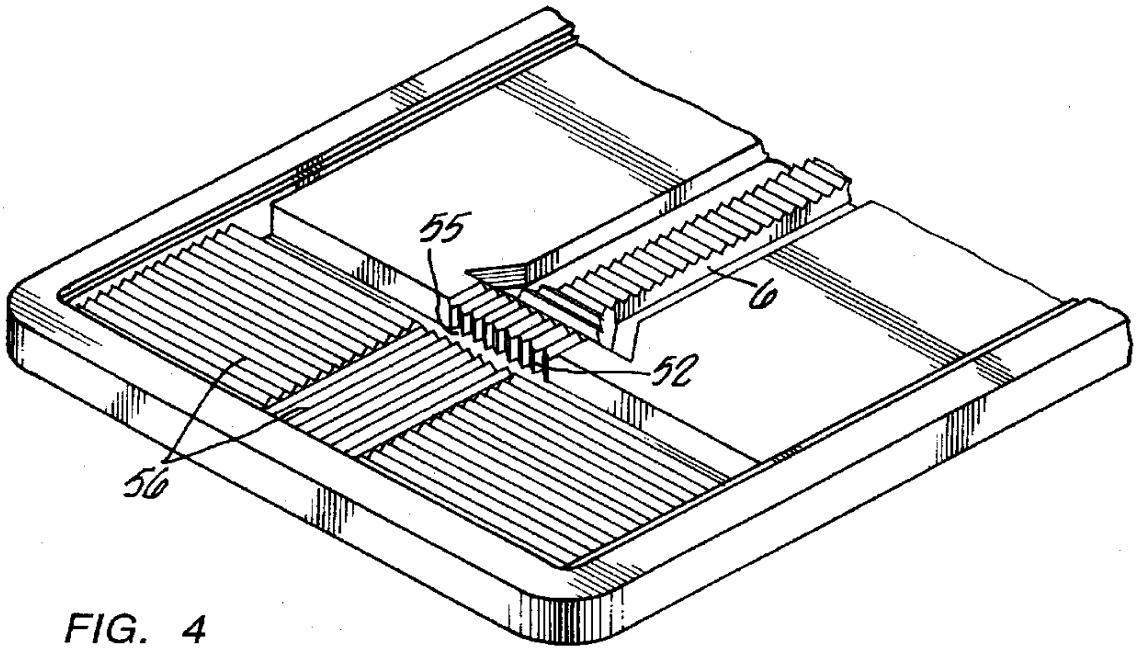


FIG. 4

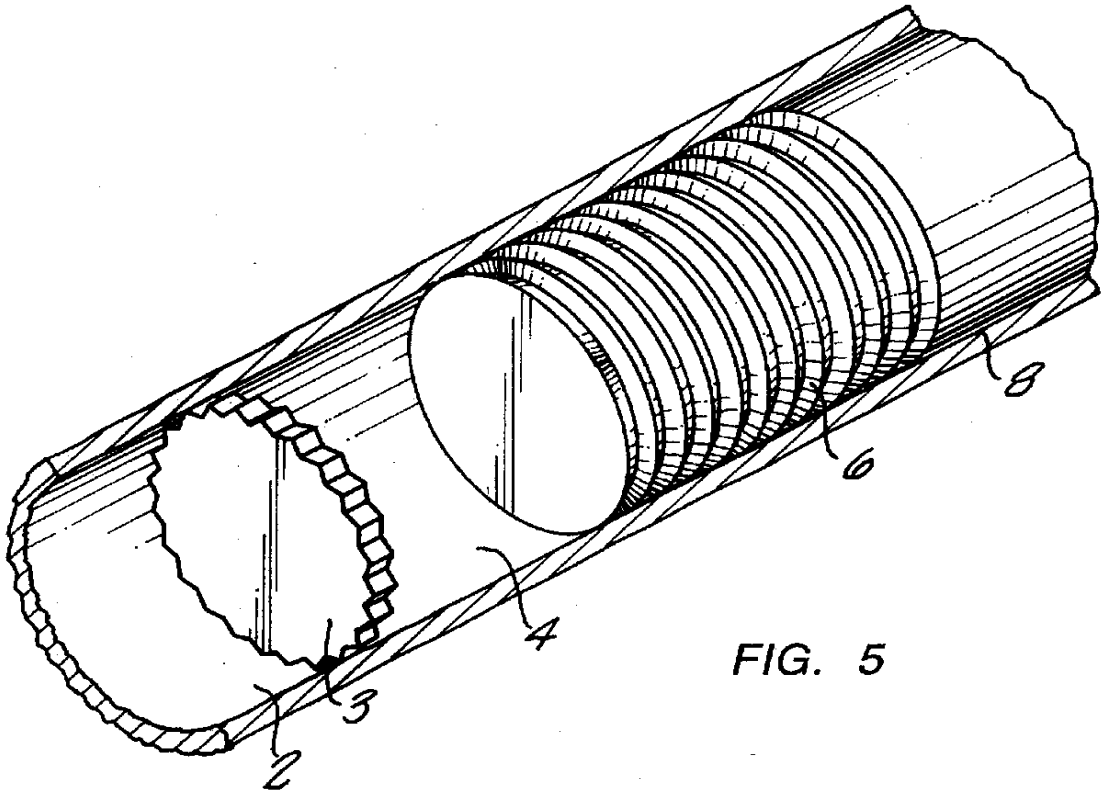


FIG. 5

DIAGNOSTIC DEVICES FOR THE CONTROLLED MOVEMENT OF REAGENTS WITHOUT MEMBRANES

FIELD OF THE INVENTION

This invention relates to devices for conducting assays, including qualitative, semi-quantitative and quantitative determinations of one or more analytes in a single test format. Unlike assay devices of the prior art, the inventive assay devices described herein do not involve the use of bibulous materials, such as papers or membranes. The inventive devices of the present invention rely on the use of defined surfaces, including grooved surfaces, and capillarity alone or in various combinations to move the test reagents. The inventive devices described herein provide means for the controlled, timed movement of reagents within the device and do not require precise pipetting steps. The concepts and devices of the present invention are especially useful in the performance of immunoassays of environmental and industrial fluids, such as water, and biological fluids and products, such as urine, blood, serum, plasma, spinal and amniotic fluids and the like.

BACKGROUND OF THE INVENTION

Over the years, numerous simplified test systems have been designed to rapidly detect the presence of a target ligand of interest in biological, environmental and industrial fluids. In one of their simplest forms, these assay systems and devices usually involve the combination of a test reagent which is capable of reacting with the target ligand to give a visual response and an absorbent paper or membrane through which the test reagents flow. Paper products, glass fibers and nylon are commonly used for the absorbent materials of the devices. In certain cases, the portion of the absorbent member containing the test reagents is brought into contact, either physically or through capillarity, with the sample containing the target ligand. The contact may be accomplished in a variety of ways. Most commonly, an aqueous sample is allowed to traverse a porous or absorbent member, such as porous polyethylene or polypropylene or membranes by capillarity through the portion of the porous or absorbent member containing the test reagents. In other cases, the test reagents are pre-mixed outside the test device and then added to the absorbent member of the device to ultimately generate a signal.

Commercially available diagnostic products employ a concentrating zone methodology. In these products, such as ICON® (Hybritech Incorporated), TESTPACK™ (Abbott Laboratories) or ACCULEVEL® (Syva Corporation), the device contains an immunosorbing or capture zone within a porous member to which a member of a specific binding pair is immobilized. The surface of the porous member also may be treated to contain one or more elements of a signal development system. In these devices, there is a liquid absorbing zone which serves to draw liquid through the immunosorbing zone, to absorb liquid sample and reagents and to control the rate at which the liquid is drawn through the immunosorbing zone. The liquid absorbing zone is either an additional volume of the porous member outside of the immunosorbing zone or an absorbent material in capillary communication with the immunosorbing zone. Many commercially available devices and assay systems also involve a wash step in which the immunosorbing zone is washed free of non-specifically bound signal generator so that the presence or amount of target ligand in the sample can be determined by examining the porous member for a signal at the appropriate zone.

The devices described herein do not use bibulous or porous materials, such as membranes and the like as substrates for the immobilization of reagents or to control the flow of the reagents through the device. A disadvantage of, for example, membranes in diagnostic devices is that on both microscopic and macroscopic scales the production of membranes is not easily reproducible. This can result in diagnostic devices which have differential properties of non-specific binding and flow characteristics. Membranes are very susceptible to non-specific binding which can raise the sensitivity limit of the assay. In the case of immunochromatographic assay formats such as those described in U.S. Pat. Nos. 4,879,215, 4,945,205 and 4,960,691, the use of membranes as the diagnostic element requires an even flow of reagents through the membrane. The problem of uneven flow of assay reagents in immunochromatographic assays has been addressed in U.S. Pat. Nos. 4,756,828, 4,757,004 and 4,883,688, incorporated herein by reference. These patents teach that modifying the longitudinal edge of the bibulous material controls the shape of the advancing front. The devices of the current invention circumvent these membrane associated problems by the use of defined surfaces, including grooved surfaces, capillarity, time gates, novel capillary means, including channels and novel fluid flow control means alone or in various combinations, all of which are constructed from non-absorbent materials. In a preferred mode of this invention, the capillary channel of the diagnostic element is composed of grooves which are perpendicular to the flow of the assay reagents. The manufacture of grooved surfaces can be accomplished by injection molding and can be sufficiently reproducible to provide control of the flow of reagents through the device.

In addition to the limitations of the assay devices and systems of the prior art, including the limitations of using absorbent membranes as carriers for sample and reagents, assay devices generally involve numerous steps, including critical pipetting steps which must be performed by relatively skilled users in laboratory settings. Accordingly, there is a need for one step assay devices and systems, which, in addition to controlling the flow of reagents in the device, control the timing of the flow of reagents at specific areas in the device. In addition, there is a need for assay devices which do not require critical pipetting steps but still perform semi-quantitative and quantitative determinations. The inventive devices and methods of this invention satisfy these needs and others by introducing devices which do not require precise pipetting of sample, which do not use absorbent members, which include novel elements called time gates for the controlled movement of reagents in the device and which are capable of providing quantitative assays.

DEFINITIONS

In interpreting the claims and specification, the following terms shall have the meanings set forth below.

Target 1 ligand—The binding partner to one or more receptors.

Ligand—Binding partner to a ligand receptor.

Ligand Analogue—A chemical derivative of the target ligand which may be attached either covalently or noncovalently to other species, for example, to the signal development element. Ligand analogue and target ligand may be the same and both are capable of

binding to the receptor.

Ligand Analogue Conjugate—A conjugate of a **1** ligand analogue and a signal development element;

Signal Development Phase—The phase containing the materials involving the signal development element to develop signal, e.g., an enzyme substrate solution.

Receptor—Chemical or biochemical species capable of reacting with or binding to target ligand, typically an antibody, a binding fragment, a complementary nucleotide sequence or a chelate, but which may be a ligand if the assay is designed to detect a target ligand which is a receptor. Receptors may also include enzymes or chemical reagents that specifically react with the target ligand.

Ligand Receptor Conjugate—A conjugate of a ligand receptor and a signal development element.

Signal Development Element—The element which directly or indirectly causes a visually or instrumentally detectable signal as a result of the assay process. Receptors and ligand analogues may be bound, either covalently or noncovalently to the signal development element to form a conjugate. The element of the ligand analogue conjugate or the receptor conjugate which, in conjunction with the signal development phase, develops the detectable signal, e.g., an enzyme.

Reaction Mixture—The mixture of sample suspected of containing target ligand and the reagents for determining the presence or amount of target ligand in the sample, for example, the ligand analogue conjugate or the receptor conjugate. As used herein the Reaction Mixture may comprise a proteinaceous component which may be the target, a component of the sample or additive (e.g., serum albumin, gelatin, milk proteins).

Ligand Complement—A specialized ligand used in labelling ligand analogue conjugates, receptors, ligand analogue constructs or signal development elements.

Ligand Complement Receptor—A receptor for ligand complement.

Ligand Analogue-Ligand Complement Conjugate—A conjugate composed of a ligand analogue, a ligand complement and a signal development element.

Capture Efficiency—The binding efficiency of the component or components in the reaction mixture, such as the ligand analogue conjugate or the receptor conjugate, to the capture zone of the diagnostic element.

Capture Zone—The area on the diagnostic element which binds at least one component of the reaction mixture, such as the **1** ligand analogue conjugate or the receptor conjugate.

Capillarity—The state of being capillary or the exhibition of capillary action. Capillarity can be affected by the solid surface or the liquid surface or both.

Biosensor—Any electrochemical, optical, electro-optical or acoustic/mechanical device which is used to measure the presence or amount of target ligands. For example, electrochemical biosensors utilize potentiometric and amperometric measurements, optical biosensors utilize absorbance, fluorescence, luminescence and evanescent waves. Acoustic/mechanical biosensors utilize piezoelectric crystal resonance, surface acoustic waves, field-effect transistors, chemical field-effect transistors and enzyme field-effect transistors.

Hydrophillic Surface—Any surface over which fluids may flow by capillarity.

Hydrophobic Surface—Any surface over which fluids cannot flow by capillarity.

Description of the Drawings FIG. **1** is a partially schematic, top perspective view of a device in accordance with the present invention.

Figure 1*a* is a partially schematic, perspective exploded view of the device showing the detail in the area of the sample addition reservoir, the sample-reaction barrier, the reaction chamber, the time gate and the beginning of the diagnostic element.

Figure 1*b* is a partially schematic, perspective exploded view of the device showing the detail in the area of the optional reagent reservoir, the sample addition reservoir, the sample-reaction barrier, the reaction chamber, the time gate and the beginning of the diagnostic element.

Figure 1*c* is a partially schematic, perspective exploded view of the device showing the detail in the area of the optional reagent reservoir in fluid contact with the sample addition reservoir and the reaction chamber.

Figure 1*d* is a partially schematic, perspective cutaway view of the flow control means.

FIG. **2** is a partially schematic, perspective view of a second device in accordance with this present invention, which may be used to add pre-mixed reaction mixtures.

FIG. **3**(*a–b*) is a partially schematic top view of the diagnostic element showing some potential placements of capture zones.

FIG. **4** is a partially schematic, perspective view of a used reagent reservoir.

FIG. **5** is a partially schematic view of embodiments of these devices which are columnar or have curved opposing surfaces.

SUMMARY OF THE INVENTION

The assay devices, assay systems and device components of this invention comprise at least two opposing surfaces disposed a capillary distance apart, at least one of which is capable of immobilizing at least one target ligand or a conjugate in an amount related to the presence or amount of target ligand in the sample from a fluid sample in a zone for controlled fluid movement to, through or away the zone. The inventive device components may be incorporated into conventional assay devices with membranes or may be used in the inventive membrane-less devices herein described and claimed. These components include flow control elements, measurement elements, time gates, elements for the elimination of pipetting steps, and generally, elements for the controlled flow, timing, delivery, incubation, separation, washing and other steps of the assay process.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to diagnostic testing devices for determining the presence or amount of at least one target ligand. FIG. **1** shows a preferred embodiment of a device **10** according to the invention. Generally, the devices of the invention have thicknesses of about 2 mm to 15 mm, lengths of about 3 cm to 10 cm and widths of about 1 cm to 4 cm. The dimensions may be adjusted depending on the particular purpose of the assay. One device of this invention, as depicted in FIG. **1**, generally illustrates some features of the inventive devices and portions of devices herein disclosed and claimed. The device **10** comprises various elements, a sample addition zone **1**, a sample

addition reservoir **2**, a sample reaction barrier **3**, a reaction chamber **4**, a time gate **5**, a diagnostic element **6**, and a used reagent reservoir **7**. The devices are comprised of capillary channels which are formed when a top member **8** is placed on the bottom member **9** a capillary distance apart and which move the reagents and sample throughout the device. The elements of the device can be used in various combinations with the diagnostic element **6** to achieve a variety of desired functions. As one skilled in the art will recognize these elements may be combined to perform one-step or multistep assays. The devices **10** may also be used in the formation of reaction mixtures for the assay process. The device **20** in FIG. **2** may be used to add pre-mixed reaction mixtures for the generation of signal which relates to the presence or amount of the target ligand.

An optional reagent chamber **17** may be incorporated into device **10** or **20** as depicted in FIG. **1***b* and FIG. **1***c*. The devices **10** and **20** may also be used with an optional fluid control means **18** as shown in FIG. **1***d*.

Features include, but are not limited to: 1) diagnostic elements which are not comprised of bibulous materials, such as membranes, 2) means to control the volume of sample or reaction mixture, 3) time gates, 4) novel capillary means, termed fingers herein and 5) novel flow control means, sometimes referred to as a "gap" herein and 6) used reagent reservoir which prevents backward flow of reagents. Those of skill in the art will appreciate that these elements are separately novel and nonobvious, and may be incorporated into diagnostic devices in various combinations and may be used with other elements known to those skilled in the art to achieve novel and nonobvious diagnostic test devices and heretofore unrealized benefits.

Each of the elements of devices **10** and **20** will be described separately, then representative descriptions of the devices of this invention will follow.

Sample Addition Zone

Referring to FIGS. **1** and **2**, the sample addition zone **1** of the devices **10** and **20** is the area where sample is introduced to the device. The sample addition zone **1** can be a port of various configurations, that is, round, oblong, square and the like or the zone can be a trough in the device.

Sample Addition Reservoir

Referring to FIGS. **1** and **2**, the sample addition reservoir **2** is an element of the device which receives the sample. Referring now to FIG. **1**, the volume of the sample addition reservoir **2** should be at least the volume of the reaction chamber **4** or greater. The sample addition reservoir **2** can be a capillary space or it can be an open trough. In addition, a filter element can be placed in or on the sample addition reservoir **2** to filter particulates from the sample or to filter blood cells from blood so that plasma can further travel through the device. In a preferred embodiment, the volume or capacity of the sample addition reservoir **2** is 1 to 5 times the volume of the reaction chamber **4**. In general, one selects a volume or capacity of this reservoir **2** such that if the excess sample is used to wash the diagnostic element **6** then enough volume of sample is needed to thoroughly remove any unbound reagents from the diagnostic element **6** arising from the assay process. This reservoir **2** may also contain certain dried reagents which are used in the assay process. For example, a surfactant can be dried in this reservoir **2** which dissolves when sample is added. The surfactant in the sample would aid in the movement of the sample and reaction mixture through the device by lowering the surface tension of the liquid. The sample addition reservoir **2** is generally in direct fluid contact with the sample-reaction barrier **3** (FIG. **1**) or the diagnostic element **6** (FIG. **2**).

Sample-Reaction Barrier

As depicted in FIG. **1**, the sample-reaction barrier **3** separates the sample in the sample addition reservoir **2** from the reaction mixture in the reaction chamber **4**. The sample-reaction barrier is desired because it provides the device with the capability of forming a precise reaction mixture volume. A precise volume of the reaction mixture is generally necessary for assays in which semi-quantitative or quantitative results are desired. Thus, a precise pipetting step of the sample to the device is not required because the sample reaction barrier forms a reaction chamber of precise volume into which the sample is capable of flowing. The sample reaction barrier **3** is desired because the reactions which take place in the reaction chamber **4** should preferably be separated from the excess sample in the sample addition reservoir **2**. The sample reaction barrier **3** comprises a narrow capillary, generally ranging from about 0.01 mm to 0.2 mm and the surfaces of the capillary can be smooth or have a single groove or a series of grooves which are parallel or perpendicular to the flow of sample. In a preferred embodiment of the sample reaction barrier **3**, now referring to FIG. **1***a*, grooves **12**, parallel to the flow of sample, are incorporated onto one surface of the device a capillary distance, for example, 0.02 mm to 0.1 mm, from the other surface. The volume of sample which fills the sample-reaction barrier **3** (FIG. **1***a*) should be kept to a minimum, from about 0.01% to 10% of the reaction chamber **4** volume so that the reagents of the reaction chamber **4** do not significantly diffuse back into the sample in the sample addition reservoir **2**. That is, the diffusion of the reaction mixture back into the excess sample should be kept to a minimum so that the chemical or biochemical reactions occurring in the reaction mixture are not substantially influenced by the excess sample in the sample addition reservoir **2**. Groove depths can range from about 0.01 mm to 0.5 mm and preferably from about 0.05 mm to 0.2 mm. When more than one groove is used for this element, the number of grooves in this element is typically between 10 and 500 grooves per cm and preferably from about 20 to 200 grooves per cm. Sample from the sample addition reservoir **2** flows over the grooves **12** by capillary action and then into the reaction chamber **4**. In a further preferred embodiment, grooves, hereafter termed "fingers" **16**, are situated in the wall of the reaction chamber **4** in fluid contact with the grooves **12** or capillary space of the sample-reaction barrier **3**. These fingers **16** are typically 0.5 mm to 2 mm wide, preferably 1 mm to 1.5 mm wide and typically 0.1 mm to 1.5 mm in depth, preferably about 0.2 to 1 mm in depth. The fingers **16** in the wall of the reaction chamber **4** aid in the capillary flow of the sample into the reaction chamber **4**. The top surface of the sample reaction barrier may also be used to immobilize reagents used in the assay process such that the sample flows over the sample reaction barrier, dissolves the reagents and moves into the reaction chamber. The movement of the sample and reagents into the reaction chamber may act as a mixing means.

Reaction Chamber

Referring to FIG. **1**, the sample moves into the reaction chamber **4** from the sample-reaction barrier **3**. The reagents of the device **10** are preferably placed in the reaction chamber **4**, for example, as dried or lyophilized powders,

such that when the sample enters the reaction chamber **4** the reagents quickly reconstitute. The volume of the reaction chamber **4** is the volume of sample which defines the reaction mixture. Thus, delivery of the sample to the device **10** at the sample addition zone **1** does not require a precise pipetting step to define the volume of the reaction mixture. Mixing features which mix the reaction mixture can also be incorporated in conjunction with the reaction chamber element **4**, such as those described in U.S. patent application Ser. No. 711,621 filed Jun. 5, 1991, hereby incorporated by reference. The sample fills the reaction chamber **4** because of capillary forces and also, potentially, because of the hydrostatic pressure exerted by the sample in the sample addition reservoir **2**. The floor of the reaction chamber 4 may be smooth or comprised of a grooved surface to aid in the movement of the sample into the reaction chamber **4**. The volume of the reaction chamber **4**, and thereby the reaction mixture, may be any volume which accommodates the reagents and which provides the desired sensitivity of the assay. The shape of the reaction chamber **4** should be such that the movement of the reaction mixture from the reaction chamber **4** is not turbulent and eddies are not formed as a result of the movement out of the reaction chamber **4**. A preferred shape of the reaction chamber **4** is shown in FIG. **1**. The depth of the reaction chamber **4** should be commensurate with the width of the chamber to accommodate the desired reaction mixture volume. The depth of the reaction chamber can range from about 0.05 mm to 10 mm and preferably from 0.1 mm to 0.6 mm. To accommodate a particular volume of the reaction chamber, the length and width of the reaction chamber should be adjusted and the depth maintained as narrow as is practical. The reaction chamber **4** is in direct fluid contact with the sample-reaction barrier **3** and the diagnostic element **6** or time gate **5**. In addition, the reaction chamber **4** may also be in direct fluid contact with an optional reagent reservoir **17** as shown in FIGS. **1***b* and l*c*.

Time Gate

Referring to FIG. **1***a*, the time gate **5** holds the reaction mixture in the reaction chamber **4** for a given period of time. The concept of the time gate is that a predominantly aqueous solution cannot pass through a hydrophobic zone until the hydrophobic zone is made hydrophilic. Furthermore, the hydrophobic zone is made hydrophilic by a component in the aqueous solution. The amount of time which is required to hold the reaction mixture in the reaction chamber **4** is relative to the assay process such that the reactions which occur in the reaction chamber **4** as a result of the assay process will reflect the presence or amount of target ligand in the sample. Thus, the time gate **5** delays the flow of the reaction mixture onto the diagnostic element **6**. The time gate **5** delays the flow of the reaction mixture by the principle that a hydrophilic liquid, such as an aqueous solution or one which has a dielectric constant of at least **40**, cannot move past a hydrophobic barrier in a capillary channel. In designing and building a time gate, one can begin with a hydrophobic surface, such as are found on native plastics and elastomers (polyethylene, polypropylene, polystyrene, polyacrylates, silicon elastomers and the like) or silicon chip surfaces or metal surfaces, either smooth, grooved or textured and a capillary is formed by an opposing surface which can be hydrophobic or hydrophilic in nature and smooth, grooved or textured. The hydrophobic surface(s) in the capillary have a microscopic surface area onto which can bind components which are generally soluble in a predominantly aqueous solution. The hydrophilic character and the concentration of the component(s) in the reaction mixture and the overall surface area of the time gate affects the mechanics of the time gate. The amount of time for which the time gate **5** holds the reaction mixture is related to the rate of binding of a component(s) from the reaction mixture to the hydrophobic barrier. The binding of the component(s) from the reaction mixture changes the hydrophobic barrier to a hydrophilic zone over which the reaction mixture can flow. For example, in a preferred embodiment, the time gate **5** can be composed of latex particles **15** (FIG. **1***a*, not drawn to scale), such as polystyrene latexes with diameters of between about 0.01 μm and 10 μm or hydrophobic polymers, such as polypropylene, polyethylene, polyesters and the like, which are introduced onto the device in the appropriate zone where the reaction mixture must travel. The component(s) in the reaction mixture which bind to the hydrophobic zone may be various proteins, polypeptides, polymers or detergents. A preferred protein is bovine serum albumin. The time delay provided by the time gate **5** depends on the concentration of the component(s) in the reaction mixture, for example, bovine serum albumin, which binds to the hydrophobic zone, for example, the surface area provided by the latex particles **15**. Another preferred embodiment of the time gate **5** utilizes polyelectrolytes which are hydrophobic and which become hydrophilic by exposure to the buffering capacity of the reaction mixture. The time gate **5** would be comprised of, for example, polyacrylic acid, which in its protonated form it is hydrophobic. The reaction mixture, if buffered above the pK_a of the polyacrylic acid, would deprotonate the acid groups and form the hydrophilic salt of the polymer. In this case, the time delay is related to the mass of polyelectrolyte and the pH and the buffering capacity of the reaction mixture.

Referring to FIG. **1**, one skilled in the art can recognize that each device **10** could incorporate one or more time gates to achieve the desired function of the device. For example, as discussed in the next section, Optional Reagent Chambers, if a sequential addition immunoassay was to be performed by the device then 2 time gates would allow 2 sequential incubation steps to be performed by the device prior to the movement of the reaction mixture to the diagnostic element. In another example, if an incubation of the reaction mixture on the capture zone or zones of the diagnostic element(s) **6** was required then a time gate(s) would be placed immediately behind the capture zone or zones. This use of the time gate may arise in cases where poor efficiency of binding of the component in the reaction mixture to the capture zone of the diagnostic element would prevail.

Optional Reagent Chambers

Referring to FIGS. **1***b* and **1***c*, the optional reagent chamber **17** is useful for the introduction of reagents into the assay process. In general, the optional reagent chamber **17** may be in direct fluid contact with the sample addition reservoir **2** via a sample reaction barrier **3** or a port the reaction chamber **4** or the diagnostic element **6**, via a sample reaction barrier **3** or a port. For example, FIG. **1***b* shows the optional reagent chamber **17** in direct fluid contact with the reaction chamber **4**. The flow of the introduced reagent may be controlled by a time gate **5**a and fingers **16** can aid in the movement of reagents into the reaction chamber **4**. Referring now to FIG. **1***c*, for example, if a sequential addition immunoassay was to be performed by the device then two time gates **5** and **5***a* would allow two sequential incubation steps to be performed

in the optional reagent chamber **17** and then in the reaction chamber **4** by the device prior to the movement of the reaction mixture onto the diagnostic element **6**. That is, sample would be applied to the sample addition reservoir **2** through the sample addition zone **1** and the sample flows over the sample reaction barrier **3** and into the optional reagent chamber **17** by the aid of fingers **16** where the first set of reactions would occur. The time gate **5***a*, after the appropriate amount of time, would allow the reagents to flow over the sample reaction barrier **3***a* and into the reaction chamber **4** by the aid of fingers **16***a* where the next set of reactions would take place. After the appropriate amount of time, the time gate **5** allows the flow of reaction mixture onto the diagnostic element **6**.

Fluid Control Means

Referring to FIG. **1***d*, the optional fluid control means **18** is designed to control the flow of the reaction mixture in the device. More specifically, the optional fluid control means **18** causes the volume of the reaction mixture to flow over the capture zone of the diagnostic element **6** at a gate which allows for an optimum capture of reagents onto the capture zone. After the volume of the reaction mixture flows over the capture zone the rate of flow of the excess reagents may be increased. The differential rate of flow of the reagents in the device is achieved by designing a gap **18** between the surfaces of the capillary space **19** of the diagnostic element **6**. The size of the gap **18** is larger than the capillary space **19** of the diagnostic element **6**. The gap **18** generally follows the capture zone or the zone where the rate of flow is required to be decreased. The gap **18** in the diagnostic element **6** thus has an associated volume. The volume of the gap **18** is filled with the reaction mixture by capillary action as it moves through the device. Since the gap **18** after the capture zone is greater than the capillary space **19** of the diagnostic element **6** a drop in capillary pressure at the beginning of the gap **18** results in a decrease in the rate of flow of the reaction mixture into the gap **18** and therefore a decrease in the rate of flow of the reaction mixture over the capture zone. Varying the size of the gap **18** changes the capillarity in the gap and thus the flow of the reaction mixture over the capture zone. In the case of immunoassays requiring a wash step to remove unbound reagents from the diagnostic element **6**, it is generally desired that the rate of flow of the wash solution over the diagnostic element **6** is faster than the rate of flow of the reaction mixture over the diagnostic element **6** because this decreases the time of the assay. The shape of the gap can take many forms. As shown in FIG. **1***d*, the gap has square corners, however, the gap can be shaped as a trapezoid or triangle which would change the rate of flow of the reaction mixture while flowing into the gap. One skilled in the art can also appreciate that for certain immunoassays a wash step is not required.

The control of the rate of flow of the reagents in the device can also be used to allow chemical reactions to take place in one zone of the device before the reagents move to another area of the device where the extent of reaction of the reagents is monitored or where further reaction may take place. For example, several fluid control means could be incorporated into a device for use in immunoassays where a sequential addition and incubation of reagents is necessary. That is, the sample comes in contact with the first reagents and the time for the reaction of the sample and first reagents is controlled by a first gap. When the first gap is filled with fluid, the reaction mixture continues to the second reagents at which time an additional chemical reaction can subsequently take place. The time required for completion of this second reaction can also be controlled by a second gap before further flow of the reaction mixture along the diagnostic element. Chemical and biochemical reactions also take place in the volume of the gap, for example, by immobilizing reagents in the gap.

Diagnostic Element

Referring to FIGS. **1** and **2**, the diagnostic element **6** is formed by opposing surfaces which are a capillary distance apart through which the reaction mixture flows and on which are placed one or more capture zones. The capture zones are comprised of reagents, such as receptors, or devices, such as biosensors which bind or react with one or more components from the reaction mixture. The binding of the reagents from the reaction mixture to the capture zones of the diagnostic element **6** is related to the presence or amount of target ligand in the sample. One or more receptors or biosensors can be placed on the diagnostic element **6** to measure the presence or amount of one or more target ligands. The receptors or biosensors can be placed in discrete zones on the diagnostic element or they can be distributed homogeneously or heterogeneously over the surface. Receptors or other chemical reagents, for example, a receptor against the signal generator can also be immobilized on the diagnostic element **6** to verify to the user that the reagents of the reaction mixture are viable and that the reaction mixture passed through the zones of the receptors or biosensors. A single receptor or biosensor can be placed over the majority of the diagnostic element **6** such that as the reaction mixture flows through the diagnostic element **6** the components from the reaction mixture bind to the surface of the diagnostic element **6** in a chromatographic fashion. Thus, the distance which the component of the reaction mixture binds would be related to the concentration of the target ligand in the sample. The reagents, such as receptors, are immobilized on the surface of the diagnostic element **6** through covalent bonds or through adsorption. A preferred embodiment is to immobilize receptor coated latex particles, for example of diameters ranging from about 0.1 μm to 5 μm. The surfaces of the diagnostic element **6** would allow the receptor coated latex particles to bind to the diagnostic element **6**. In a preferred embodiment, the receptors bind to the surface of the diagnostic element through electrostatic, hydrogen bonding and/or hydrophobic interactions. Electrostatic, hydrogen bonding and hydrophobic interactions are discussed, for example, in Biochemistry 20, 3096 (1981) and Biochemistry 29, 7133 (1990). For example, the diagnostic element **6** can be treated with a plasma to generate carboxylic acid groups on the surface. The receptor coated latex particles are preferably applied to the diagnostic element **6** in a low salt solution, for example, 1–20 mM, and at a pH which is below the isoelectric point of the receptor. Thus, the negative character of the carboxylic acid groups on the diagnostic element **6** and the positive charge character of the receptor latex will result in enhanced electrostatic stabilization of the latex on the diagnostic element **6**. Hydrogen bonding and hydrophobic interactions would also presumably contribute to the stabilization and binding of the receptor latex to the diagnostic element **6**.

In an additional embodiment of the diagnostic element, now referring to FIG. **5**, the diagnostic element **6** is a cylindrical surface which may be composed of grooves. When the diagnostic element is composed of grooves, the grooves generally run perpendicular to the flow of the reaction mixture. A capillary space is formed around the

diagnostic element by a round tube which is generally clear; thus, the surface of the diagnostic element and the opposing surface of the tube are a capillary distance apart. The capillary formed allows the flow of the reaction mixture over the round diagnostic element **6**. Generally, the reaction mixture would travel up against gravity or down with gravity through the cylindrical capillary space. The capture zones of the round diagnostic element **6** can be placed in discrete zones or over the entire length of the diagnostic element **6**. The capture zones may also circle the diameter of the diagnostic element **6** or may be applied to only a radius of the diagnostic element **6**. The reaction mixture may be delivered to the diagnostic element **6** through the tube **8**. Furthermore, the cylindrical volume of the tube **8** may be used as a reaction chamber **4** and a disc shaped sample reaction barrier **3** with grooves on its perimeter may also be inserted to form the reaction chamber **4** and the sample addition reservoir **2**. From this discussion, now referring to FIG. **1** and **2**, one skilled in the art can also appreciate that the flat diagnostic element **6** may also be curved such that the curvature is a radius of a circle.

One skilled in the art can appreciate that various means can be used for the detection of signal at the capture zone of the diagnostic element. In the case of the use of biosensors, such as, for example, a piezoelectric crystal, the piezoelectric crystal onto which would be immobilized a receptor, would be the capture zone and the response generated by binding target ligand would be generally reflected by an electrical signal. Other types of detection means include, but are not limited to visual and instrumental means, such as spectrophotometric and reflectance methods. The inventive features of the diagnostic element described herein allows for improved capture efficiencies on surfaces over which a reaction mixture flows and that various means for detection may be used by one skilled in the art.

The surfaces of the capillaries in the device are generally hydrophilic to allow flow of the sample and reaction mixture through the device. In a preferred embodiment the surface opposing the diagnostic element **6** is hydrophobic such that the reaction mixture repels this surface. The repulsion of reaction mixture to the surface opposing the diagnostic element **6** forces the reaction mixture to the surface where capture occurs, thus improving the capture efficiency of the components of the reaction mixture to the capture zone. In another preferred embodiment, the diagnostic element **6** is hydrophilic but the areas adjacent to,the diagnostic element **6** are hydrophobic, such that the reagents of the assay are directed through only the hydrophilic regions of the diagnostic element. One skilled in the art will recognize that various techniques may be used to define a hydrophilic diagnostic element or zone, such as plasma treatment of hydrophobic surfaces using masks which shield the surfaces, except for the diagnostic element, from the treatment or by application of hydrophobic adhesives to hydrophilic surfaces to define a diagnostic element or by the use of viscous hydrophobic compounds, such as an oil or a grease.

The surfaces of the diagnostic element **6** may be smooth or grooved or grooved and smooth. Groove depths can range from about 0.005 mm to 0.5 nun and preferably from about 0.02 mm to 0.3 mm. The number of grooves is typically between about 10 and 500 grooves per cm and preferably between 50 and 300 grooves per cm. In a preferred mode as shown in FIGS. **1** and **2**, one surface of the diagnostic element **6** is grooved and the grooves are perpendicular to the flow of the reaction mixture and the opposing surface is smooth. In another embodiment, one surface of the diagnostic element **6** is grooved at the capture zone and the areas adjacent to the capture zone are smooth. The opposing surface of the diagnostic element **6** may be smooth or may be grooved, for example, the grooves of each surface intermesh. The positioning of the grooves of the diagnostic element perpendicular to the flow of the reaction mixture is beneficial in that the flow of the reaction mixture through the diagnostic element **6** occurs in an organized manner with a distinct, straight front dictated by the grooves in the capillary space. In addition, when one surface is in close proximity, for example 1 μm to 100 μm, to the peaks of the grooves then the capture efficiency of the components from the reaction mixture can be enhanced. The enhancement of capture efficiency at the capture zones in grooved diagnostic elements as compared to smooth surface elements may be related to the movement of the reaction mixture in the capillary space; that is, in the case of the grooved surface the reaction mixture is forced to move over the peak of the groove and into the trough of the next groove. Thus, a finer grooved surface, that is, more grooves per cm, would provide a better capture efficiency than a coarser grooved surface. The reaction mixture is thus driven closer to the surface of the grooved diagnostic element than it would be if both surfaces were smooth. Also, the close proximity of the surfaces decreases the volume of the bulk reaction mixture above the grooved surface of the diagnostic element and therefore decreases the diffusion distance of the components which bind to the diagnostic element. The proximity of the surfaces of the diagnostic element should minimize the volume of reaction mixture in the diagnostic element at the capture zone without blocking the capillary flow through the element. The capillary space can be defined by a variety of ways, for example, machining the surfaces to the appropriate tolerances or using shims between the surfaces. The surfaces of the diagnostic element can be parallel or non-parallel. In the latter case, the flow rate of the reagents through the diagnostic element will not be uniform throughout the length. A preferred embodiment is to maintain the surfaces of the diagnostic element approximately parallel. The surfaces of the diagnostic element can be made from materials, such as plastics which are capable of being milled or injection molded, for example, polystyrene, polycarbonate, polyacrylate and the like or from surfaces of copper, silver and gold films upon which are adsorbed various long chain alkanethiols as described in J.Am. Chem. Soc. 1992, 114, 1990–1995 and the references therein. In this latter example, the thiol groups which are oriented outward can be used to covalently immobilize proteins, receptors or various molecules or biomolecules which have attached maleimide or alkyl halide groups and which are used to bind components from the reaction mixture for determining the presence or amount of the target ligand.

Referring to FIGS. **3***a* and **3***b*, the zones of immobilization of one or more receptors or the placement of biosensors at the capture zone **17** on the diagnostic element **6** can take many forms. For example, if the target ligand is very low in concentration in the sample then one would desire that all of the reaction mixture pass over the zone of immobilized receptor or biosensor to obtain the best signal from the given volume of reaction mixture. In this case, the placement of the reagents or biosensors on the diagnostic element **6** at the capture zones **17** could, for example, resemble that shown in FIG. **3***a*. If the target ligand in the sample is high in concentration and the sensitivity of the analytical method is not an issue then the placement of the receptors or biosensors at the capture zones **17** could, for example, resemble that in FIG. **3***b*. One skilled in the art can appreciate that the placement of receptors or biosensors on the diagnostic

element is a function of the sensitivity requirements of the analytical method.

One or more diagnostic elements can comprise a device. The reaction mixture may be applied to a device with multiple diagnostic elements. In addition, the sample may be applied to the device and then separated into different reaction chambers, each with separate diagnostic elements. The capture zone can be various geometrical symbols or letters to denote a code when the sample is positive or negative for the target ligand. One skilled in the art will recognize the useful combinations of the elements of this invention.

Used Reagent Reservoir

Referring to FIGS. **1** and **2**, the used reagent reservoir **7** receives the reaction mixture, other reagents and excess sample from the diagnostic element **6**. The volume of the used reagent reservoir **7** is at least the volume of the sample and extra reagents which are added to or are in the device. The used reagent reservoir **7** can take many forms using an absorbent, such as a bibulous material of nitrocellulose, porous polyethylene or polypropylene and the like or the used reagent reservoir can be comprised of a series of capillary grooves. In the case of grooves in the used reagent reservoir **7**, the capillary grooves can be designed to have different capillary pressures to pull the reagents through the device or to allow the reagents to be received without a capillary pull and prevent the reagents from flowing backwards through the device. The size and quantity of the grooved capillaries determine the volume and capillarity of the used reagent reservoir **7**. In a preferred embodiment, as shown in FIG. **4**, the fingers **52** at the end of the diagnostic element **6** are in fluid contact with a capillary space **55** and the capillary space **55** is in fluid contact with a grooved or textured capillary space **56**. The depth of the grooves or textured surface can be, for example, about 0.1 mm to 0.6 mm, preferably about 0.3 mm to 0.5 mm and the density can range from about 5 to 75 grooves per cm and preferably about 10 to 50 grooves per. cm. Referring to FIG. **4**, the reagents of the device move to the fingers **52** at the end of the diagnostic element **51** and into the capillary channel **55**. The reagents either partially or completely fill the capillary space **55** and then come in contact with the grooved or textured surface **56**. The width of the capillary space **55** is generally about 1 mm to 3 mm and the depth is generally about 0.1 mm to 2 mm. The length of the capillary space **55** should be sufficient to be in fluid contact with the grooved or textured surface **56**. The grooved or textured surface **56** partially or completely pulls the reagents from the capillary channel **55** depending on the rate of delivery of the reagents into the capillary space **55** from the diagnostic element **51**. When the flow of reagents is complete in the device, the grooved or textured surface **56** has greater capillarity than the capillary channel **55** and the reagents are removed from the capillary channel **55** by the grooved or textured surface **56**. In addition, the reverse flow of the reagents from the grooved or textured surface is not preferred because the capillarity in the grooved or textured surface **56** holds the reagents and prevents their backward flow. One skilled in the art can recognize from these inventive features that the arrangement of grooves or a used reagent reservoir within the device can be adapted to a variety of desired objectives.

The Description of the One-Step Assay Device

The elements of the device which have been described individually can be assembled in various ways to achieve the desired function. The term "one-step" implies that one manual action is required to achieve the assay result, for example, adding sample to the device is one step. In the case of the device performing a one-step assay which involves both a timed incubation of reagents and a wash step, the wash solution is excess sample and the assay device is built with the elements in fluid communication using the sample addition reservoir, the sample-reaction barrier, the reaction chamber, the time gate, the diagnostic element and the used reagent reservoir as depicted in FIG. **1**. The devices are generally about 3 cm to 10 cm in length, 1 cm to 4 cm in width and about 2 mm to 15 mm thick. Typically, a top member with smooth surfaces is placed onto a bottom member which has a surface onto which are built the elements stated above. The relationship of the elements are as depicted in FIG. **1**. The reagents required for performing the assay are immobilized or placed in the respective elements. The surfaces are brought together, a capillary distance apart, and in doing so, the regions of the sample addition reservoir, the sample reaction barrier, the reaction chamber, the time gate, the diagnostic element, the gap and the used reagent reservoir are all formed and are capable of functioning together. Also, the surfaces are brought together such that the opposing surfaces touch to form and seal the sample addition reservoir, the reaction chamber, and the used reagent reservoir.

When performing a qualitative, non-competitive assay on one or more target ligands, the signal producing reagents, which could include, for example, a receptor specific for the target ligand adsorbed to a colloidal metal, such as a gold or selenium sol, are placed on the sample reaction barrier or in the reaction chamber in dried or lyophilized form. Another receptor for each target ligand is immobilized onto the surface of the diagnostic element at the capture zone. The time gate is positioned generally on the diagnostic element between the reaction chamber and the capture zones by the placement of, for example, a surfactant-free polystyrene suspension onto the device in an amount which dictates the desired incubation time. The incubation time is usually the amount of time for the reactions to come to substantial equilibrium binding. The assay is then performed by addition of sample to the sample addition reservoir of the device. The sample moves over the sample-reaction barrier, into the reaction chamber by the aid of the fingers and dissolves the reagents in the reaction chamber to form the reaction mixture. The reaction mixture incubates for the amount of time dictated by the time gate. The excess sample remaining in the sample addition reservoir and reaction mixture in the reaction chamber are in fluid communication but are not in substantial chemical communication because of the sample-reaction barrier. Thus, the reaction chamber defines the volume of the reaction mixture. The reaction mixture then moves past the time gate and onto the diagnostic element and over the capture zones. The complex of receptor conjugate and target ligand formed in the reaction mixture binds to the respective receptor at the capture zone as the reaction mixture flows over the capture zones. The reaction mixture may also flow over a positive control zone, which can be for example, an immobilized receptor to the signal development element. As the reaction mixture flows through the diagnostic element and into the used reagent reservoir by the aid of the fingers, the excess sample flows behind the reaction mixture and generally does not substantially mix with the reaction mixture. The excess sample moves onto the diagnostic element and removes the receptor conjugate which did not bind to the capture zone. When sufficient excess sample washes the diagnostic element, the signal at the capture zones can be interpreted visually or instrumentally. Referring to FIG. **1***d*, in a preferred mode of the above

description, the reaction mixture moves onto the diagnostic element **6**, over the capture zone or zones and then the reaction mixture proceeds into a capillary gap **18**. The capillary gap **18** generally has less capillarity than that of the diagnostic element **6**. The capillary space **19** of the diagnostic element **6** is generally smaller than the capillary space of the gap **18**. The volume of the capillary gap **18** generally approximates the volume of the reaction mixture such that the capillary gap **18** fills slowly with the reaction mixture and once filled, the capillarity of the remaining portion of the diagnostic element **6** or used reagent reservoir is greater than the capillarity of the gap **18** resulting in an increased rate of flow to wash the diagnostic element **6**. As one skilled in the art can appreciate, the gap **18** can be formed in the top member **8** or in the bottom member **9** or a combination of both members **8** and **9**.

In the case of the device performing a one-step assay which does not involve a timed incubation step but does involve a wash step in which the wash solution is excess sample, the assay device is built with the elements in fluid communication using the sample addition reservoir, the sample-reaction barrier, the reaction chamber, the diagnostic element and the used reagent reservoir. The assay reagents are used as described above for the non-competitive qualitative assay. The assay device without the time gate allows the reaction mixture to flow onto the diagnostic element without an extended incubation time. The capillary flow of the reaction mixture and the excess sample are as described above.

The optional reagent chamber is incorporated into the device in the case of the device performing a one-step assay with the introduction of an additional assay reagent into or after the reaction mixture or the introduction of a wash solution which flows behind the reaction mixture through the device. The optional reagent chamber may be in fluid contact with any element of the device and is generally in fluid contact with the reaction chamber. When in fluid contact with, for example, the reaction chamber, the optional reagent chamber and the reaction chamber may be separated by a time gate. Various reagents may be dried or lyophilized in the optional reagent chamber, such as detergents for a washing step or reagents which are sequentially provided to the diagnostic element after the reaction mixture.

In the case of performing one-step, non-competitive, quantitative assays the reagents as described above for the non-competitive, qualitative assay may apply. The device is comprised of the elements, sample addition reservoir, sample-addition barrier, reaction chamber, time gate, diagnostic element and used reagent reservoir. In this case, the capture zone of the diagnostic element is generally the entire diagnostic element. That is, the capture zone is a length of the diagnostic element onto which the receptor conjugate binds. The receptor conjugate binds along the length of the capture zone in proportion to the amount of target ligand in the sample. The device of the present invention is preferred for this quantitative assay because of the high efficiency of capture of the reagents, for example, the binding of a complex of target ligand and receptor conjugate to an immobilized receptor to the target ligand on the capture zone, and because the movement of the reaction mixture over the diagnostic element proceeds with a sharp front. The receptors on the capture zone sequentially become saturated with the complex of target ligand and receptor conjugate as the reaction mixture moves over the length of the capture zone. The length of the diagnostic element containing bound conjugate then determines the concentration of the target ligand. Those skilled in the art will recognize the format of this type of immunoassay as a quantitative immunochromatographic assay as discussed in U.S. Pat. Nos. 4,883,688 and 4,945,205, hereby incorporated by reference.

In the case of the device performing a one-step, qualitative, competitive assay which involves both a timed incubation of reagents and a wash step and the wash solution is excess sample, the assay device is built with the elements in fluid communication using the sample addition reservoir, the sample-reaction barrier, the reaction chamber, the time gate, the diagnostic element and the used reagent reservoir. When performing a qualitative competitive assay on one or more target ligands, the conjugate is composed of, for example, a ligand analogue coupled to signal development element, such as a gold or selenium sol. The conjugate and receptor for each target ligand are placed in the reaction chamber in dried or lyophilized form, for example, in amounts which are taught by U.S. Pat. Nos. 5,028,535 and 5,089,391, hereby incorporated by reference. Another receptor for each target ligand is immobilized onto the surface of the diagnostic element at the capture zone. The time gate is positioned generally on the diagnostic element between the reaction chamber and the capture zones as described previously. The incubation time is usually the amount of time for the reactions to come to substantial equilibrium binding. The assay is then performed by addition of sample to the device. The sample moves over the sample-reaction barrier and into the reaction chamber, dissolves the reagents to form the reaction mixture and incubates for the time dictated by the time gate. The excess sample and reaction mixture are in fluid communication but not in substantial chemical communication because of the sample-reaction barrier. The reaction mixture then moves onto the diagnostic element and over the capture zones. The ligand analogue conjugate binds to the respective receptor or receptors at the capture zone or zones. As the reaction mixture flows over the diagnostic element and into the used reagent reservoir, the excess sample flows behind the reaction mixture and generally does not substantially mix with the reaction mixture. The excess sample moves onto the diagnostic element and removes conjugates which do not bind to the capture zone or zones. When sufficient excess sample washes the diagnostic element the results at the capture zones can be interpreted visually or instrumentally. In a preferred mode of the above invention, the reaction mixture moves onto the diagnostic element, over the capture zone or zones and then the reaction mixture proceeds into a capillary gap. The capillary gap has less capillarity than that of the diagnostic element. The volume of the capillary gap generally approximates the volume of the reaction mixture such that the capillary gap fills slowly with the reaction mixture and once filled, the capillarity of the remaining portion of the diagnostic element or used reagent reservoir is greater resulting in an increased rate of flow of excess sample to wash the diagnostic element.

In another aspect of the one-step, competitive assay, the reaction mixture is composed of ligand analogue-ligand complement conjugate to each target ligand and receptors adsorbed to latex particles with diameters of, for example, 0.1 μm to 5 μm to each target ligand, in appropriate amounts, for example, as taught by U.S. Pat. Nos. 5,028,535 and 5,089,391. The ligand complement on the conjugate can be any chemical or biochemical which does not bind to the receptors for the target ligands. The assay is begun by addition of sample to the device. Sample fills the reaction chamber and is incubated for a time which allows the reagents to come to substantial equilibrium binding. The reaction mixture flows over the time gate and onto or into a filter element to prevent ligand analogue-ligand complement

conjugates which have bound to their respective receptor latexes from passing onto the diagnostic element. Typical filter elements can be composed of nitrocellulose, cellulose, nylon, and porous polypropylene and polyethylene and the like. Thus, only the ligand analogue-ligand complements conjugate which were not bound by the receptor latex will pass onto the diagnostic element. The receptor to the ligand complement of the conjugate is immobilized on the diagnostic element at the capture zone and binds the conjugate. A wash step may not be required because the filter removes the conjugate bound to latex; however, the excess sample or a wash solution from the optional reagent chamber may be used to wash the diagnostic element.

In the case of a one-step quantitative, competitive assay, the receptor to the ligand analogue conjugate or the ligand complement of the conjugate is immobilized onto the diagnostic element as described previously for the one-step quantitative, non-competitive assay. Thus, the concentration of the target ligand in the sample is visualized by the distance of migration on the diagnostic element of the conjugate.

The Device as a Diagnostic Element

The diagnostic element of the device can be utilized with a sample addition means to perform a separation step of bound and unbound conjugates. An example of this type of device which has a sample addition means, a diagnostic element and a used reagent reservoir is depicted in FIG. 2. For example, in the case of a non-competitive assay, at least one receptor conjugate is incubated with sample which is suspected of containing at least one target ligand in a suitable vessel and this reaction mixture is applied to the sample addition zone of the device. The reaction mixture then flows onto the diagnostic element and over the capture zone of, for example, immobilized receptor to the target ligand. When target ligand is present in the sample, the target ligand-receptor conjugate complex binds to the receptor on the capture zone. If the signal development element is an enzyme, then either a substrate for the enzyme which produces a visual color or a wash solution followed by a substrate is next added to the device. Excess reagents flow to the used reagent reservoir. The presence or amount of each target ligand in the sample is then determined either visually or instrumentally.

In the case of a competitive immunoassay, for example as taught by U.S. Pat. Nos. 5,028,535 and 5,089,391, herein incorporated by reference, the diagnostic element may be used to separate bound and unbound ligand analogue conjugates such that the unbound ligand analogue conjugates bind to the receptors of the diagnostic element in proportion to the presence or amount of target ligand in the sample.

One skilled in the art can appreciate that all formats of immunoassays or gene probe assays which require a separation step of free and bound conjugates or the separation of free of bound reagents which subsequently leads to the ability to detect a signal can utilize the inventive features of the diagnostic element. One skilled in the art can also recognize that the inventive elements of this invention, namely, the fingers, the sample reaction barrier, the reaction chamber, the time gate, the diagnostic element, the fluid control means and the used reagent reservoir can be used separately or in various combinations and in conjunction with other devices not described here. For example, the sample reaction barrier with fingers and the reaction chamber can be used in conjunction with devices incorporating porous members, such as membranes to deliver precise volumes of reagents to the porous member. The time gate can also be incorporated into the aforementioned devices or the time gate may be used alone in conjunction with devices incorporating porous members. The fluid control means can also be used in devices incorporating porous members to control the rate of flow of reagents through the porous member.

Experimental Procedures

Example 1

Preparation of anti-βhCG Antibody-Colloidal Gold Conjugate

Colloidal gold with an average diameter of 45 nm was prepared according to the method of Frens, Nature, Physical Sciences, 241, 20 (1973). The colloidal gold conjugate was prepared by first adding 5.6 ml of 0.1M potassium phosphate, pH 7.58, dropwise with rapid stirring to 50 ml of colloidal gold. Anti β-subunit monoclonal antibody to hCG (Applied Biotech, San Diego, Calif.; 1 ml of 4.79 mg/ml in phosphate buffered saline, 0.02% sodium azide, pH 7) was added in a bolus to the colloidal gold with rapid stirring. After complete mixing the stirring was stopped and the solution was incubated at room temperature for 1 h. Polyethylene glycol (average molecular weight=20,000) was added (0.58 ml) as a 1% solution to the colloidal gold solution and the solution was mixed. The colloidal gold solution was subjected to centrifugation at 27,000 g and 5° C. for 20 min. The supernatant was removed and each pellet was washed twice by resuspension and centrifugation with 35 ml of 10 mM potassium phosphate, 2 mM potassium borate, 0.01% polyethylene glycol (average molecular weight=20,000), pH 7. After the final centrifugation, the pellet was resuspended in 0.5 ml of the wash buffer. The gold conjugate was diluted for the assay of hCG into a buffered solution containing 10 mg/ml bovine serum albumin at pH 8.

Example 2

Preparation of anti-αhCG Antibody Latex

Surfactant-free polystyrene particles (Interfacial Dynamics Corp., Portland, Oreg.; 0.106 ml of 9.4% solids, 0.4 μm) was added while vortexing to anti α-subunit hCG monoclonal antibody (Applied Biotech, San Diego, Calif.; 0.89 ml of 6.3 mg/ml in 0.1M 2-(N-morpholino) ethane sulfonic acid, (MES), pH 5.5) and the suspension was incubated at room temperature for 15 min. The suspension was subjected to centrifugation to pellet the latex particles. The pellet was washed three times by centrifugation and resuspension of the pellet with 10 mM MES, 0.1 mg/ml trehalose, pH 5.5. The final pellet was resuspended in the wash buffer at a solids concentration of 1%.

Example 3

Preparation of Goat Anti-Mouse Latex

Surfactant-free polystyrene particles (Interfacial Dynamics Corp , Portland, Oreg.; 0.11 ml of 9.4% solids 0.6 μm) were added while vortexing to goat IgG antibody against mouse IgG (Jackson ImmunoResearch Laboratories, Inc.; 0.89 ml of 0.34 mg/ml in 0.1M MES, pH 5) and the suspension was incubated at 45° C. for 2 h. The suspension was subjected to centrifugation to pellet the latex particles. The pellet was washed three times by centrifugation and resuspension of the pellet with 10 mM MES, 0.2 mg/ml

trehalose, pH 5.5. The final pellet was resuspended in the wash buffer at a solids concentration of 1%.

Example 4

Preparation of the One-Step Device for a Qualitative hCG Assay

A one-step device made of plastic was built having an 80 to 100 μl sample addition reservoir, a 20 μl reaction chamber and a 40 μl used reagent reservoir. This device is designed for applying samples of about 20 μl to 100 μl, but the reaction chamber is fixed at 20 μl. In cases where a larger reaction mixture volume is required for the desired assay, then the reaction chamber would be increased to that volume and the sample addition reservoir would be about 2 to 4 times the volume of the reaction chamber volume. The devices were plasma treated to graft functional groups which create a hydrophilic surface. Those skilled in the art will recognize that the plasma treatment of plastic is performed in a controlled atmosphere of a specific gas in a high frequency field. The gas ionizes, generating free radicals which react with the surface. The sample addition reservoir was shaped as a trapezoid with dimensions of 14 mm and 7 mm for the parallel sides and 7 mm for the other sides with a depth of 0.49 mm. The sample addition reservoir was adjacent to the sample reaction barrier. The sample-reaction barrier was 1.5 mm long and 7 mm wide including grooves running parallel to the flow of the sample at a density of 50 grooves per cm and a depth of 0.1 mm. In the case of sample volumes larger than 20 to 80 μl, the width of the reaction barrier and thereby the reaction chamber could be increased to accommodate the desired flow rate but the groove size or density could remain as indicated. The fingers in the walls of the reaction chamber and the used reagent reservoir were 1 mm wide and 0.4 mm deep with 7 fingers in each wall of the reaction chamber and the used reagent reservoir. The reaction chamber volume was 20 μl. The reaction chamber was shaped as a trapezoid with dimensions of 7 mm and 3.5 mm for the parallel sides and 7.1 mm for the other sides with depths of 0.56 mm for 20 μl reaction chambers. The diagnostic element was about 2.5 cm long, 2 mm wide and 1 mm from the base of the device including grooves running perpendicular to the flow of reaction mixture at a density of 100 grooves per cm and a depth of 0.05 mm. In the case of a time gate on the diagnostic element, the time gate was positioned on the diagnostic element immediately adjacent to the reaction chamber. The width of the diagnostic element could be increased to increase the flow of the reaction mixture to the desired rate past the capture zones. The anti-αhCG antibody latex (1 μl) and the goat anti-mouse latex (1 μl) were applied to the diagnostic element of the devices approximately 1.5 cm apart. The anti-BhCG antibody colloidal gold conjugate (10 μl) was pipetted into the trough of the reaction chamber. The devices were placed under vacuum for about 15 min. to dry the reagents. The used reagent reservoir had the shape of a trapezoid with dimensions of 7 mm and 15 mm for the parallel sides and 8 mm for the other sides with a depth of 0.5 mm. Referring to FIG. 4, in a preferred (best mode) embodiment of the used reagent reservoir, the reaction mixture moved to a capillary space **55** (1.25 mm long, 27.5 mm wide and 0.48 mm deep) from the diagnostic element **6**, aided by fingers **52** (1 mm wide and 0.4 mm deep with 7 fingers), and then into a grooved capillary structure (13.6 mm long, 25.4 mm wide, 0.61 mm deep with a density of 16 grooves per cm). The outer walls and the top surface of the walls of the sample addition reservoir and the reaction chamber had applied a thin coating of silicon grease to prevent the leakage of the reagents from the reservoir and chamber of the assembled device. The capillary spaces in the devices were then formed by placing a clear plastic polycarbonate sheet on top of the device. The plastic sheet was held to the opposing surface with binder clips. The clear plastic sheet had a sample port above the sample addition reservoir for the introduction of sample.

Example 5

Qualitative One-step Assay for hCG

The devices described in Example 4 were used for the qualitative one-step assay for hCG. The assay times for the devices without the time gates were about 5 to 10 min. A urine solution (60 μl) containing 0, 50, 200 and 500 mIU hCG/ml was added to the sample reservoir of the devices. The sample moved into the reaction chamber, dissolved the colloidal gold conjugate and the reaction mixture moved onto the diagnostic element over the anti-hCG latex and goat anti-mouse IgG latex capture zones. The reaction mixture moved into the used reagent reservoir and the excess sample washed the diagnostic element. The color density of the capture zones for hCG was measured instrumentally using a Minolta Chroma Meter CR 241 at 540 nm. A red color was visible for samples containing hCG and not visible for the sample without hCG at the capture zones for hCG. The ΔE* values for the 0, 50, 200 and 500 mIU/ml were 0, 7.78, 12.95 and 20.96, respectively, and for the positive control (goat anti-mouse IgG) zones a distinctive red bar was observed with a ΔE* of about 35.

Example 6

Qualitative One-Step Assay for hCG Using a Time Gate

Devices as described in Example 4 were prepared with the addition of the time gate. The time gate was formed on the diagnostic element which is in contact with the reaction mixture in the reaction chamber. The time gate was prepared by adding 1 μl of 2% solids of surfactant-free, sulfated latex, 1.0 μm, (Interfacial Dynamics Corp., Portland, Oreg.). The other reagent latexes and gold conjugate were also added to the devices and dried as described in Example 5. Clear plastic sheets were placed on the devices and sample (about 60 μl) containing 0, 50, 200 and 500 mIU hCG/ml was added to the devices. The sample moved into the reaction chamber, dissolved the colloidal gold conjugate and the reaction mixture remained in the reaction chamber for about 8 to 10 min, whereas in devices without time gates the reaction mixture remained in the reaction chamber for 5 sec to 15 sec. The proteinaceous components of the reaction mixture, which may be present in the sample and which was added as a component of the reaction mixture, namely, bovine serum albumin, bound to the latex particles of the time gate and changed the hydrophobic surface of the time gate into a hydrophilic surface. Other proteins, such as gelatin, serum albumins, immunoglobulins, enzymes and the like and polypeptides and hydrophilic polymers will also function to bind to the hydrophobic zone. The gradual transformation of the hydrophobic surface to a hydrophilic surface, which resulted through binding of the proteinaceous components of the reaction mixture to the latex particles allowed the reaction mixture to flow over the area of the time gate. In control experiments in which protein, namely bovine serum albumin, was not added to the reaction mixture, flow of the reaction mixture over the time gate and onto the diagnostic element did not occur during the time (5 h) of the experiment. This control experiment showed that the urine sample

alone did not contain sufficient protein or components which bind to the applied latex of the time gate to allow a change in the hydrophobic character of the time gate. In the event that the components in the sample should only be used to cause the transformation of the hydrophobic time gate to a hydrophilic one for the reaction mixture to flow, then one would be required to lower the mass and total surface area of the latex applied to the time gate to an extent which would allow flow of the reaction mixture over the time gate in an appropriate amount of time. The reaction mixture then moved onto the diagnostic element over the anti-hCG latex and goat anti-mouse IgG latex capture zones. The reaction mixture moved into the used reagent reservoir and the excess sample washed the diagnostic element. The color density of the capture zones for hCG was measured instrumentally using a Minolta Chroma Meter CR 241. A red color was visible for samples containing hCG and not visible for the sample without hCG at the capture zones for hCG. The ΔE* values for the 0, 50, 200 and 500 mIU/ml were 0, 6.51, 13.14 and 18.19, respectively. A red color bar was visible at the goat anti-mouse IgG capture zones of each device.

Example 7

Qualitative One-Step Assay for hCG Using a Flow Control Means

Devices as described in Example 4 were prepared with the addition of the optional flow control means. The optional flow control means or "gap" was placed behind the capture zone for hCG gold conjugate on the diagnostic element. The gap between the two surfaces was 0.38 mm, the length of the gap was 13.2 mm and the width of the gap on the top member was 9 mm; however, the effective width of the gap was the width of the diagnostic element (2 mm). This gap volume above the diagnostic element was about 10 μl which was, in this case, half the volume of the reaction chamber. The anti-hCG and the goat anti-mouse latexes and gold conjugate were added to the device and dried as described in Example 5. Clear plastic sheets of polycarbonate having a gap in one surface were placed on the devices with the gap facing the diagnostic element. Sample (about 60 μl) containing 0 and 200 mIU hCG/ml was added to the devices. The sample moved into the reaction chamber, dissolved the colloidal gold conjugate and the reaction mixture then moved onto the diagnostic element over the anti-hCG latex. The reaction mixture then entered the gap which was immediately behind the capture zone of anti-hCG latex. The flow rate over the capture zone slowed while the reaction mixture moved over the capture zone and filled the gap. The time for the 10 μl reaction mixture to fill the gap was about 12 min to 16 min, whereas with devices without the optional flow control means, the times were about 1 min to 3 min. for the reaction mixture to pass over the capture zone. When the reaction mixture filled the gap, the reaction mixture then moved into the narrow capillary of the diagnostic element and over the goat anti-mouse capture zone. The reaction mixture moved into the used reagent reservoir and the excess sample washed the diagnostic element. The color density of the capture zones for hCG was measured instrumentally using a Minolta Chroma Meter CR 241. A red color was visible for samples containing hCG and not visible for the sample without hCG at the capture zones for hCG. The ΔE* values for the 0 and 200 mIU/ml were 0 and 16.12. The δE*, value of the hCG capture zone for the device without the flow control means for the 200 mIU/ml sample was 16.32. A red color bar was visible at the goat anti-mouse IgG capture zones of each device.

Example 8

Preparation of the Diagnostic Element for Multi-step Assays

A device was built comprising a sample addition reservoir and a diagnostic element. The devices were plasma treated to graft functional groups which create a hydrophilic surface. The sample addition reservoir had dimensions of 12 mm long, 6 mm wide and 0.05 mm deep. The diagnostic element was about 5.5 cm long, 1.3 mm wide and 1 mm from the base of the device and included grooves running perpendicular to the flow of reaction mixture at a density of 100 grooves per cm and a depth of 0.05 mm. In the case of qualitative assays, the antibody latex (1 μl) was applied to the diagnostic element, covering the entire width and 1 cm length of the diagnostic element. In the case of an immunochromatographic assay, the antibody latex (6 μl) was applied to the entire width and length of the diagnostic element. The devices were placed under vacuum for about 1 h to dry the reagents. The capillary spaces in the device were then formed by placing a clear plastic polystyrene sheet on top of the device. The plastic sheet was held to the opposing surface with binder clips.

Example 9

Assay for hCG Using the Diagnostic Element

The diagnostic element described in Example 8 was used for the assay of hCG. Urine samples (20 μl) containing 0, 50, 200 and 500 mIU/ml hCG were added to tubes containing anti-BhCG antibody colloidal gold conjugate (2 μl). The tubes were vortexed and the reaction mixtures were incubated for 5 min at room temperature. The reaction mixtures (20 μl) were applied in 10 μl aliquots to the sample addition reservoir of the device. The reaction mixture flowed onto the diagnostic element from the sample reservoir and over the capture zone. An absorbent at the end of the capture zone removed the used reagent from the diagnostic element. The color density of the capture zones for hCG was measured instrumentally using a Minolta Chroma Meter CR 241. A red color was visible for samples containing hCG and not visible for the sample without hCG at the capture zones for hCG. The ΔE* values for the 0, 50, 250 and 500 mIU/ml were 0.00, 1.24, 3.16 and 5.56, respectively.

Example 10

Synthesis of meta-Nitrophencyclidine

To an ice cooled solution of phencyclidine hydrochloride (5 g, 1.8×10^{-2} mol) in concentrated sulfuric acid (9 ml) was added dropwise, and with stirring, fuming nitric acid (2 ml). The reaction mixture was stirred in an ice-water bath for 1 hour and then poured onto crushed ice/ water. The mixture was made basic with 10N sodium hydroxide (50 ml) to pH12 and extracted with diethyl ether (2×100 ml). The combined organic layers were washed with water (2×100 ml), dried over anhydrous magnesium sulfate, filtered and evaporated under vacuum. The residue was treated with methyl -alcohol (20 ml) and heated on a hot water bath (80° C.) until solute dissolved. The flask was covered with aluminum foil (product is light sensitive) and the solution was allowed to stir at room temperature overnight when a yellow solid precipitated. The solid was collected by filtration and dried under vacuum to afford 3.0 g (58%) of m-nitrophencyclidine as fine yellow crystals which were protected from light: mp 81°–82° C.

Example 11

Synthesis of meta-Aminophencyclidine

To a stirring solution of m-nitrophencyclidine (3.0 g, 0.4×10^{-3} mol) in methyl alcohol (150 ml) was added, under a flow of argon, 10% palladium-carbon (0.5 g) followed by

ammonium formate (4.0 g, 6.3×10^{-2} mol). The reaction mixture was stirred at room temperature for 2 hours after which time the catalyst was removed by filtration and the solvent was evaporated under vacuum. The residue was treated with 1N potassium hydroxide solution (30 ml) and extracted with diethyl ether (2×50 ml). The combined organic extracts were washed with water (50 ml), dried over anhydrous magnesium sulfate, filtered and evaporated under vacuum. The residue was dissolved in hexane (20ml) and the solution was stirred at room temperature overnight when a white solid precipitated. The solid was collected by filtration and dried under vacuum to afford 1.4 g (52%) of m-aminophencyclidine: mp 121°–122° C.

Example 12

Synthesis of Acetylthiopropionic Acid

To a stirred solution of 3-mercaptopropionic acid (7 ml, 0.08 moles) and imidazole (5.4 g, 0.08 moles) in tetrahydrofuran (THF, 700 ml) was added dropwise over 15 minutes, under argon, a solution of 1-acetyl imidazole (9.6 g, 0.087 moles) in THF (100 ml). The solution was allowed to stir a further 3 hours at room temperature after which time the THF was removed in vacuo. The residue was treated with ice-cold water (18 ml) and the resulting solution acidified with ice-cold concentrated HCl (14.5 ml) to pH 1.5–2. The mixture was extracted with water (2×50 ml), dried over magnesium sulfate and evaporated. The residual crude yellow oily solid product (10.5 g) was recrystallized from chloroform-hexane to afford 4.8 g (41% yield) acetylthiopropionic acid as a white solid with a melting point of 44°–45° C.

Example 13

Synthesis of meta-Acetylthiopropionamide Phencyclidine

To a stirring solution of m-aminophencyclidine (1.4 g, 5.4×10^{-3} mol) and acetylthiopropionic acid (0.87 g, 5.8×10^{-3} mol) in anhydrous tetrahydrofuran (7 ml) was added dicyclohexylcarbodiimide (1.19 g, 5.8×10^{-3} mol). The flask was purged with argon and the solution stirred at room temperature for 2 hours. The mixture was filtered from insoluble dicyclohexylurea and evaporated under vacuum. The residual solid was recrystallized from chloroform/hexane to afford 1.5 g (71%) of m-acetylthiopropionamide phencyclidine as a white crystalline solid: mp 152°–4° C.

Example 14

Synthesis of meta-3-MerCaptopropionamide Phencyclidine

meta-Acetylthiopropionamide phencyclidine (0.01 g, 2.57×10^{-5} mol) was dissolved in 1.29 ml 0.12M potassium carbonate in 80% methanol/20% water (v/v). The solution sat at room temperature for 5 min and then 0.2 ml 0.5M potassium phosphate, pH 7, was immediately added and the solution was adjusted to pH 7–7.5 with hydrochloric acid (1N). The title compound in solution was used as is to react with BSA-SMCC.

Example 15

Preparation of Phencyclidine Analogue Attached to Bovine Serum Albumin (BSA-PCPI)

Bovine serum albumin (BSA, 3.5 ml of 20 mg/ml) was reacted with succinimidyl 4-(N-maleimidomethyl)- cyclohexane-1-carboxylate (SMCC, Pierce Chemical Co.) by adding a solution of 6.7 mg SMCC in 0.3 ml acetonitrile and stirring the solution at room temperature for 1 h while maintaining the pH between 7 and 7.5 with 1N potassium hydroxide. The protein was separated from unreacted compounds by gel filtration chromatography in 0.1M potassium phosphate, 0.02M potassium borate, 0.15M sodium chloride, pH 7.0. The meta-3-mercaptopropionamide phencyclidine (0.2 ml of 13 mM) was added to the BSA-maleimide (2 ml at 8.2 mg/ml) and the solution was stirred at room temperature for 4 h. The solution was then dialyzed 3 times against 1000 ml of 10 mM MES, pH 5.5. Recover 1.8 ml BSA-PCP at 8 mg/ml.

Example 16

Preparation of Phencyclidine Analogue Colloidal Gold Conjugate

A solution (4.7 ml) containing BSA (22 mg) and BSA-PCP (5.6 mg) in 10 mMMES, pH 5.5 was added in a bolus to colloidal gold (105 ml) in 10 mM MES, pH 5.5 with rapid stirring. After complete mixing the stirring was stopped and the solution was incubated at room temperature for 1 h. The colloidal gold conjugate was subjected to diafiltration against 50 mM potassium phosphate, 10 mM potassium borate, pH 7, using a tangential flow device (Sartorius Easy Flow, molecular weight cutoff was 100,000) to remove BSA and BSA-PCP which was not bound to colloidal gold. The gold conjugate was diluted for the assay of PCP into a buffered solution containing 10 mg/ml bovine serum albumin at pH 7.5.

Example 17

Preparation of anti-Phencyclidine Antibody Latex

Surfactant-free polystyrene particles (Interfacial Dynamics Corp., Portland, Oreg.; 0.074 ml of 9.4% solids, 0.4 μm) was added while vortexing to anti-phencyclidine monoclonal antibody (0.926 ml of 5.86 mg/ml in 0.1M MES, pH 5) and the suspension was incubated at 45° C. for 2 h. The suspension was subjected to centrifugation to pellet the latex particles. The pellet was washed three times by centrifugation and resuspension of the pellet with 10 mM MES, 0.1 mg/ml trehalose, pH 5.5. The final pellet was resuspended in the wash buffer at a solids concentration of 1

Example 18

Preparation of Latex-Immobilized Affinity-Purified Goat IqG Antibody Against the Fc Fragment of Mouse IgG (Goat anti-mouse Fc latex)

Affinity-purified goat anti-mouse (Fc (Immunosearch) and polystyrene latex particles (sulfated, 1.07 μm) (Interfacial Dynamics) were incubated separately at 45° C. for one hour, the antibody solution being buffered with 0.1M 2-(N-morpholino) ethane sulfonic acid at pH 5.5. While vortexing the antibody solution, the suspension of latex particles was added to the antibody solution such that the final concentration of antibody was 0.3 mg/ml and the solution contained 1% latex solids. The suspension was incubated for 2 hours at 45° C. prior to centrifugation of the suspension to pellet the latex particles. The latex pellet was resuspended in 1% bovine serum albumin in phosphate-buffered-saline (PBS) and incubated for one hour at room temperature. Following centrifugation to pellet the latex, the pellet was washed three times by resuspension in PBS and centrifugation. The final pellet was resuspended in PBS containing 0.1% sodium azide at pH 7.0 at a latex concentration of 1% solids.

Example 19

Assay for Phencyclidine Using the Diagnostic Element

The diagnostic element described in Example 8 was used for the assay of phencyclidine (PCP). Urine samples (133 μl) containing 0, 100, 200 and 300 ng/ml PCP were added to

tubes containing a lyophilized buffer formulation (containing 10 mM potassium phosphate, 150 mM sodium chloride and 10 mg/ml BSA, pH 8) and phencyclidine analogue colloidal gold conjugate (4 μl) was added and the solution was vortexed. Anti-PCP antibody (2.8 μl of 0.1 mg/ml) was added to each tube and the solutions were vortexed and incubated at room temperature for 5 min. Goat anti-mouse Fc latex (50 ml of a 1% suspension) was added to the tubes, the tubes were vortexed and incubated at room temperature for 10 min. The solutions were then filtered to remove the complex of the PCP analogue gold conjugate: anti-PCP antibody:goat anti-mouse latex from the reaction mixture using a Gelman Acrodisc® 3 syringe filter (0.45 μm). The filtrates of the reaction mixtures (20 μl) were applied to the diagnostic elements described in example 8. The reaction mixture flowed onto the diagnostic element from the sample reservoir and over the capture zone. An absorbent tissue placed 1 cm after the capture zone removed the used reagent from the diagnostic element. The color density of the capture zones was measured instrumentally using a Minolta Chroma Meter CR **241**. The ΔE^* values for the 0, 100, 200 and 300 ng/ml samples were 0.69, 9.28, 14.04 and 21.6, respectively.

Although the foregoing invention has been described in some detail by way of illustration and example, it will be obvious that certain changes or modifications may be practiced within the scope of the appended claims. As used herein, references to "preferred" embodiments refer to best modes for practicing the invention.

I claim:

1. Diagnostic assay device for detecting at least on target ligand in an aqueous fluid sample, said device comprising:

i. a sample addition reservoir;

ii. a sample reaction barrier between said sample addition reservoir and said reaction chamber;

iii. means for fluid flow from a first capillary in said sample reaction barrier (ii) to a second capillary at a reaction chamber (iv), said first capillary having a greater capillarity than said second capillary, and a wall substantially perpendicular to fluid flow in said first capillary, said wall located at the interface between said first capillary and said second capillary, said means comprising grooves on said wall in said second capillary, said grooves each with an end thereof in contact with fluid flow and being substantially perpendicular to fluid flow, and having widths of between 0.5 mm to 2 mm wide and 0.1 mm to 1.5 mm in depth;

iv. reaction chamber containing at least one conjugate for said target ligand;

v. diagnostic element capable of immobilizing for detecting at least one target ligand in at least one zone;

vi. time gate for delaying fluid flow from said reaction chamber (iv) to said diagnostic element (v) for a preselected time at least sufficient to allow said fluid sample to dissolve said conjugate to form a reaction mixture, said time gate located between said reaction chamber and said diagnostic element, said diagnostic element adapted to receive fluid flow from said reaction chamber through said time gate, said time gate comprising three distinct zones including a first hydrophilic zone, a hydrophobic zone and a second hydrophilic zone, said hydrophobic zone located between said first and second hydrophilic zones, and having a component therein which is capable of binding at least one aqueous soluble component present in said fluid at a rate which changes said hydrophobic zone to a sufficiently hydrophilic surface to delay fluid flow into said second hydrophilic zone, which comprises said diagnostic element (v);

vii. used reagent reservoir.

2. Diagnostic assay device for detecting at least on target ligand in an aqueous fluid sample, said device comprising:

i. a sample addition reservoir;

ii. a sample reaction barrier between said sample addition reservoir of said reaction chamber;

iii. means for fluid flow from a first capillary in said sample reaction barrier (ii) to a second capillary at a reaction chamber (iv), said first capillary having a greater capillarity than said second capillary, and a wall substantially perpendicular to fluid flow in said first capillary, said wall located at the interface between said first capillary and said second capillary, said means comprising grooves on said wall in said second capillary, said grooves each with an end thereof in contact with fluid flow and being substantially perpendicular to fluid flow, and having widths of between 0.5 mm to 2 mm wide and 0.1 mm to 1.5 mm in depth;

iv. reaction chamber containing at least one conjugate for said target ligand;

vi. time gate for delaying fluid flow from said reaction chamber (iv) to said diagnostic element (v) for a preselected time at least sufficient to allow said fluid sample to dissolve said conjugate to form a reaction mixture, said time gate located between said reaction chamber and said diagnostic element, said diagnostic element adapted to receive fluid flow from said reaction chamber through said time gate, said time gate comprising three distinct zone including a first hydrophilic zone, a hydrophobic zone and a second hydrophilic zone, said hydrophobic zone located between said first and second hydrophilic zones, and having a component therein which is capable of binding at least one aqueous soluble component present in said fluid at a rate which changes said bydrophobic zone to a sufficiently hydrophilic surface to delay fluid flow into said second hydrophilic zone, which comprises said diagnostic element (v);

v. diagnostic element capable of immobilizing for detecting at least one conjugate in an amount related to the amount of target ligand in a fluid sample in at least one zone;

vii. used reagent reservoir.

3. Diagnostic assay device for detecting at least on target ligand in an aqueous fluid sample, said device comprising:

i. a sample addition reservoir;

ii. a sample reaction barrier between said sample addition reservoir and said reaction chamber;

iii. means for fluid flow from a first capillary in said sample reaction barrier (ii) to a second capillary at a reaction chamber (iv), said first capillary having a greater capillarity than said second capillary, and a wall substantially perpendicular to fluid flow in said first capillary, said wall located at the interface between said first capillary and said second capillary, said means comprising grooves on said wall in said second capillary, said grooves each with an end thereof in contact with fluid flow and being substantially perpendicular to fluid flow, and having widths of between 0.5 mm to 2 mm wide and 0.1 mm to 1.5 mm in depth;

iv. reaction chamber containing at least one conjugate for said target ligand;

v. diagnostic element capable of immobilizing for detect-

ing at least one target ligand in at least one zone;

vi. time gate for delaying fluid flow from said reaction chamber (iv) to said diagnostic element (vi) for a preselected time at least sufficient to allow said fluid sample to dissolve said conjugate to form a reaction mixture, said time gate located between said reaction chamber and said diagnostic element, said diagnostic element adapted to receive fluid flow from said reaction chamber through said time gate, said time gate comprising three distinct zone including a first hydrophilic zone, a hydrophobic zone and a second hydrophilic zone, said hydrophobic zone located between said first and second hydrophilic zones, and having a component therein which is capable of binding at least one aqueous soluble component present in said fluid at a rate which changes said hydrophobic zone to a sufficiently hydrophilic surface to delay fluid flow into said second hydrophilic zone, which comprises said diagnostic element (v):

vii. means for controlling fluid flow to used reagent reservior (viii)

viii. used reagent reservoir.

4. Diagnostic assay device for detecting at least one target ligand in an aqueous fluid sample, said device comprising:

i. a sample addition reservoir;

ii. a sample reaction barrier between said sample addition reservoir and said reaction chamber;

iii. means for fluid flow from a first capillary in said sample reaction barrier (ii) to a second capillary at a reaction chamber (iv), said first capillary having a greater capillarity than said second capillary, and a wall substantially perpendicular to fluid flow in said first capillary, said wall located at the interface between said first capillary and said second capillary, said means comprising grooves on said wall in said second capillary, said grooves each with an end thereof in contact with fluid flow and being substantially perpendicular to fluid flow, and having widths of between 0.5 mm to 2 mm wide and 0.1 mm to 1.5 mm in depth;

iv. reaction chamber containing at least one conjugate for said target ligand;

v. diagnostic element capable of immobilizing for detecting at least one conjugate in an amount related to the amount of target ligand in a fluid sample in at least one zone;

vi. time gate for delaying fluid flow from said reaction chamber (iv) to said diagnostic element (v) for a preselected time at least sufficient to allow said fluid sample to dissolve said conjugate to form a reaction mixture, said time gate located between said reaction chamber and said diagnostic element, said diagnostic element adapted to receive fluid flow from said reaction chamber through said time gate, said time gate comprising three distinct zones including a first hydrophilic zone, a hydrophobic zone and a second hydrophilic zone, said hydrophobic zone located between said first and second hydrophilic zones, and having a component therein which is capable of binding at least one aqueous soluble component present in said fluid at a rate which changes said hydrophobic zone to a sufficiently hydrophilic surface to delay fluid flow into said second hydrophilic zone, which comprises said diagnostic element (v);

vii. means for controlling fluid flow to used reagent reservoir (viii);

viii. used reagent reservoir.

5. Device of claim **1** or **2** or **3** or **4** optimally having a reagent chamber in fluid contact with said reaction chamber.

6. Device of claim **1** or **2** or **3** or **4** optimally having a reagent chamber in fluid contact with said reaction chamber and said sample addition reservoir.

7. Device of claim **1** or **2** or **3** or **4** wherein at least one signal producing element and at least one receptor capable of combining with said target is contained in said reaction chamber.

8. Device of claim **1** or **2** or **3** or **4** wherein at least one signal producing element and at least one receptor capable of combining with said target is contained on said sample reaction barrier.

9. Device of claim **1** or **2** or **3** or **4** in which said diagnostic element is a grooved surface.

10. Device of claim **1** or **2** or **3** or **4** wherein at least one signal producing element capable of combining with said target is contained in said reaction chamber.

11. Device of claim **1** or **2** or **3** or **4** wherein at least one signal producing element capable of combining with said target is contained on said sample reaction barrier.

12. Diagnostic assay device for detecting a target ligand in a fluid sample, said device comprising:

i. a reaction chamber containing at least one conjugate for said target ligand, and a fluid introducing means for said fluid sample;

ii. a time gate located between said reaction chamber (i) and said zone (iii) for delaying fluid flow to said zone (iii) for a preselected time at least sufficient to allow said fluid sample to dissolve said conjugate to form a reaction mixture, said time gate comprising three distinct zones including a first hydrophilic zone, a hydrophobic zone and a second hydrophilic zone, said hydrophobic zone located between said first and second bydrophilic zones, and having a component therein which is capable of binding at least one aqueous soluble component present in said fluid at a rate which changes said hydrophobic zone to a sufficiently hydrophilic surface to delay fluid flow into said second hydrophilic zone, which comprises said diagnostic element (vi); and

iii. at least one zone containing at least one immobilized receptor for detecting each desired target ligand.

13. Device of claim **12** in which said zone (iii) is located within a capillary space in said second hydrophilic zone, through which all of said reaction mixture flows.

14. Diagnostic assay device for detecting a target ligand in a fluid sample, said device comprising:

i. a sample addition reservoir;

ii. time gate for delaying fluid flow from said sample addition reservoir (i) to a diagnostic element (iii) for a preselected time, said time gate located between said sample addition reservoir and said diagnostic element, said diagnostic element adapted to receive fluid flow from said reaction chamber through said time gate, said time gate comprising three distinct zones including a first hydrophilic zone, a hydrophobic zone and a second hydrophilic zone, said hydrophobic zone located between said first and second hydrophilic zones, and having a component therein which is capable of binding at least one aqueous soluble component present in said fluid at a rate which changes said hydrophobic zone to a sufficiently hydrophilic surface to delay fluid flow into said second hydrophilic zone, which comprises said diagnostic element (iii);

iii. diagnostic element capable of receiving fluid flow from said sample addition reservoir, and of immobilizing for detecting at least one conjugate in an amount related to the amount of target ligand in a fluid sample in at least one zone.

15. Device of claim **1**, **2**, **3**, **4** or **14** in which said diagnostic element is located within a capillary space in said second hydrophilic zone, through which all of said reaction mixture flows.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 5,458,852
DATED : OCTOBER 17, 1995
INVENTOR(S) : KENNETH F. BUECHLER

It is certified that error appears in the above-indentified patent and that said Letters Patent is hereby corrected as shown below:

COLUMN 9, LINE 22: Change "gate" to --rate--.
COLUMN 10, LINE 23: After the word "element" insert --6--.
COLUMN 11, LINE 59: Change "nun" to --mm--.
COLUMN 25, LINE 30: Change "on" to --one--.
COLUMN 26, LINE 4: Change "on" to --one--.
COLUMN 26, LINE 47: Change "on" to --one--.

Signed and Sealed this

Twenty-seventh Day of February, 1996

Attest:

Bruce Lehman

Attesting Officer

BRUCE LEHMAN

Commissioner of Patents and Trademarks

US006019944A

United States Patent [19]

Buechler

[11] **Patent Number: 6,019,944**

[45] **Date of Patent: *Feb. 1, 2000**

[54] **DIAGNOSTIC DEVICES AND APPARATUS FOR THE CONTROLLED MOVEMENT OF REAGENTS WITHOUT MEMBRANES**

[75] Inventor: **Kenneth F. Buechler**, San Diego, Calif.

[73] Assignee: **Biosite Diagnostics, Inc.**, San Diego, Calif.

[*] Notice: This patent is subject to a terminal disclaimer.

[21] Appl. No.: **08/447,895**

[22] Filed: **May 23, 1995**

Related U.S. Application Data

[60] Division of application No. 08/065,528, May 19, 1993, abandoned, which is a continuation-in-part of application No. 07/887,526, May 21, 1992, Pat. No. 5,458,852.

[51] **Int. Cl.**[7] **G01N 33/48**

[52] **U.S. Cl.** **422/58**; 422/61; 422/73; 422/102; 436/165; 436/177

[58] **Field of Search** 422/55, 56, 58, 422/72, 73, 82.05, 100, 102; 436/69, 180, 165, 170, 177, 518, 524, 531

[56] **References Cited**

U.S. PATENT DOCUMENTS

4,233,029	11/1980	Columbus	422/58 X
4,426,451	1/1984	Columbus	436/518
4,756,828	7/1988	Litman et al.	435/7
4,757,004	7/1988	Houts et al.	435/7
4,879,215	11/1989	Weng et al.	435/7
4,883,688	11/1989	Houts et al.	427/285
4,906,439	3/1990	Grenner	422/56
4,945,205	7/1990	Litman et al.	219/121
4,960,691	10/1990	Gordon et al.	435/7
4,963,498	10/1990	Hillman	436/69
5,006,309	4/1991	Khalil et al.	422/56
5,023,054	6/1991	Sato et al.	422/82.09
5,028,535	7/1991	Buechler et al.	435/7.1
5,051,237	9/1991	Grenner et al.	422/56
5,087,556	2/1992	Ertinghausen	422/56 X
5,089,391	2/1992	Buechler et al.	435/7.1
5,137,808	8/1992	Ullman et al.	422/56 X
5,458,852	10/1995	Buechler	422/58

FOREIGN PATENT DOCUMENTS

0212314	4/1987	European Pat. Off. .
0348006	12/1989	European Pat. Off. .
0418765	3/1991	European Pat. Off. .

OTHER PUBLICATIONS

Myer, D., Surfaces, Interfaces, and Colloids: Principles and Applications, 1991, pp. 94–99.

Cambridge Dictionary of Science and Technology, Walker, editor, 1988 (reprinted 1990), p. 131.

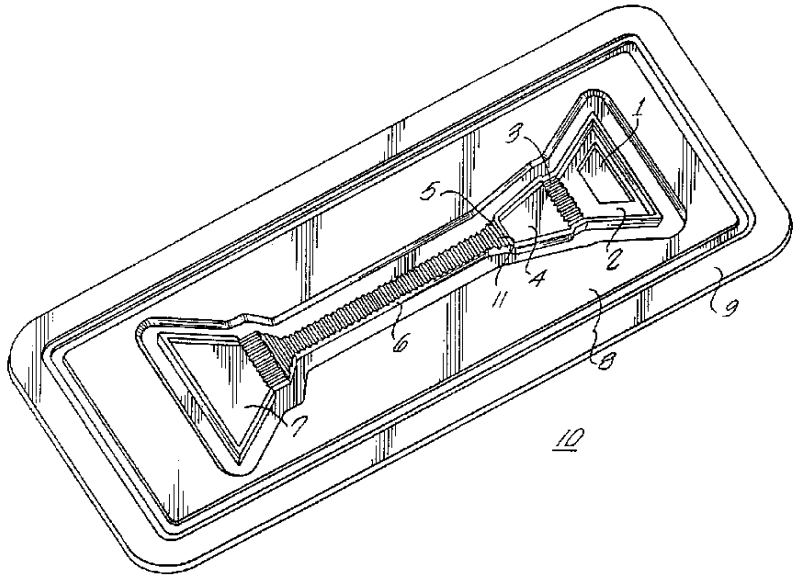
Primary Examiner—Lyle A. Alexander

Attorney, Agent, or Firm—Lyon & Lyon LLP

[57] **ABSTRACT**

The assay devices, assay systems and device components of this invention comprise at least two opposing surfaces disposed a capillary distance apart, at least one of which is capable of immobilizing at least one target ligand or a conjugate in an amount related to the presence or amount of target ligand in the sample from a fluid sample in a zone for controlled fluid movement to, through or away the zone. The inventive device components may be incorporated into conventional assay devices with membranes or may be used in the inventive membrane-less devices herein described and claimed. These components include, flow control elements, measurement elements, time gates, elements for the elimination of pipetting steps, and generally, elements for the controlled flow, timing, delivery, incubation, separation, washing and other steps of the assay process.

50 Claims, 9 Drawing Sheets



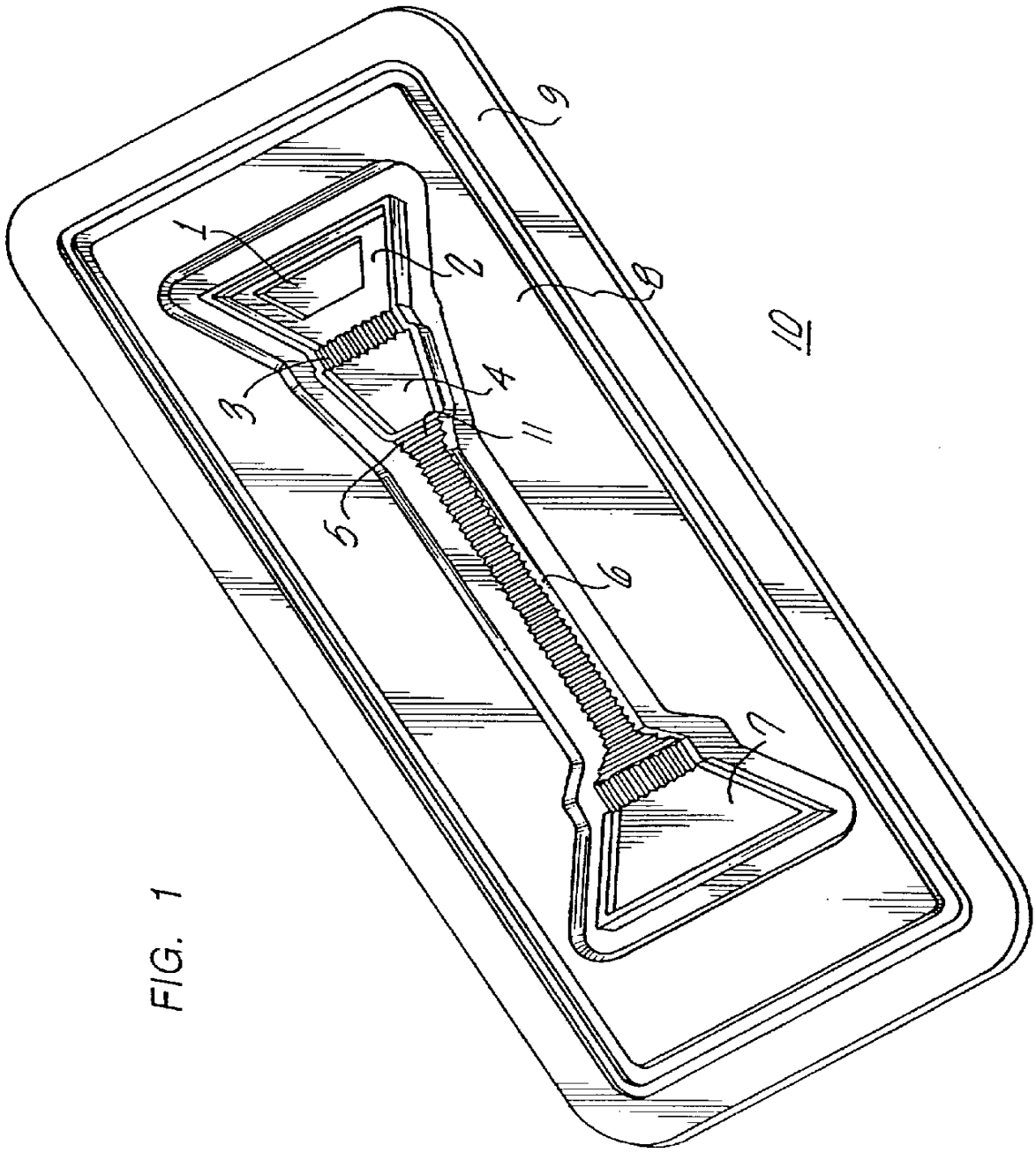


FIG. 1

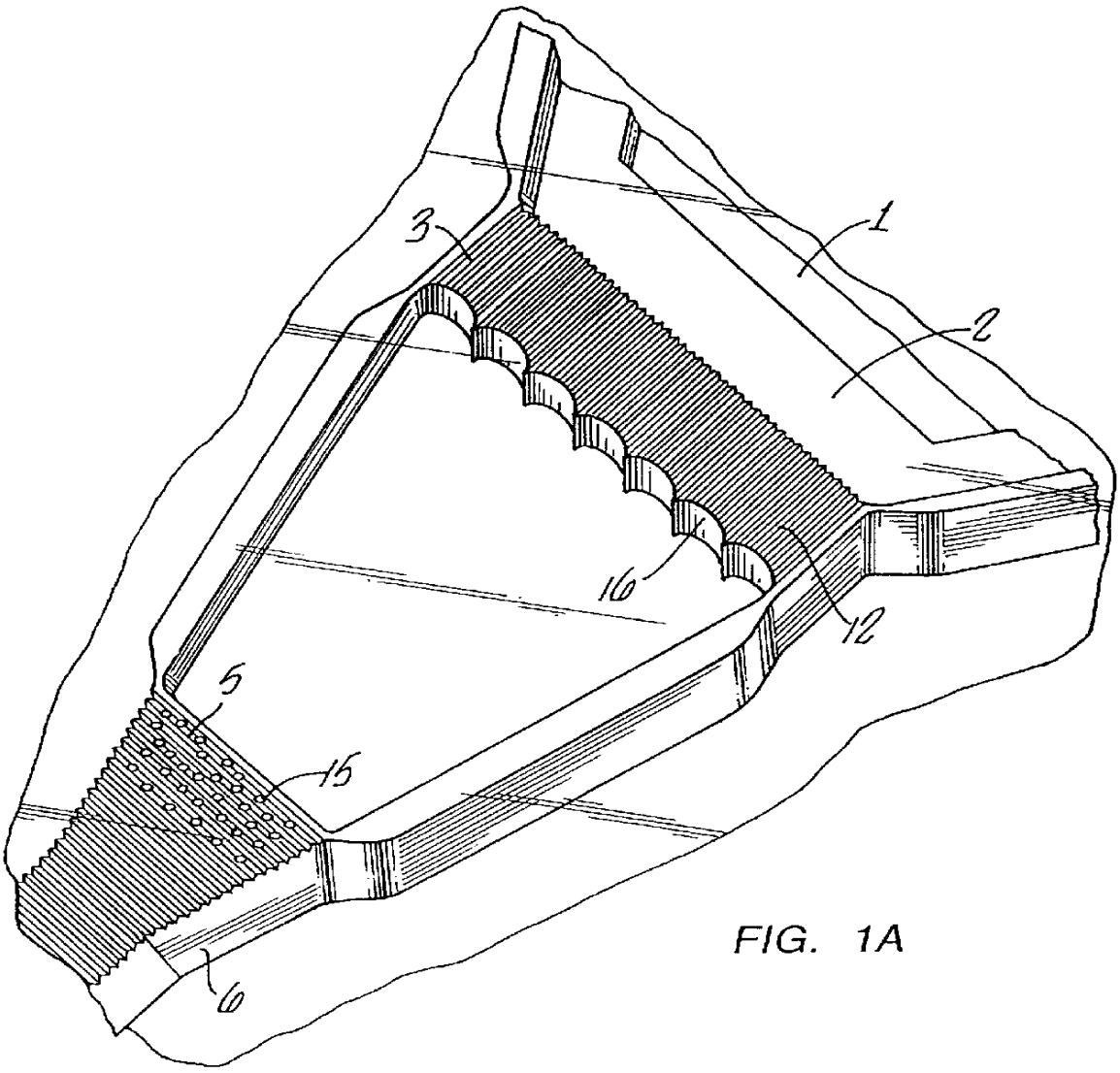


FIG. 1A

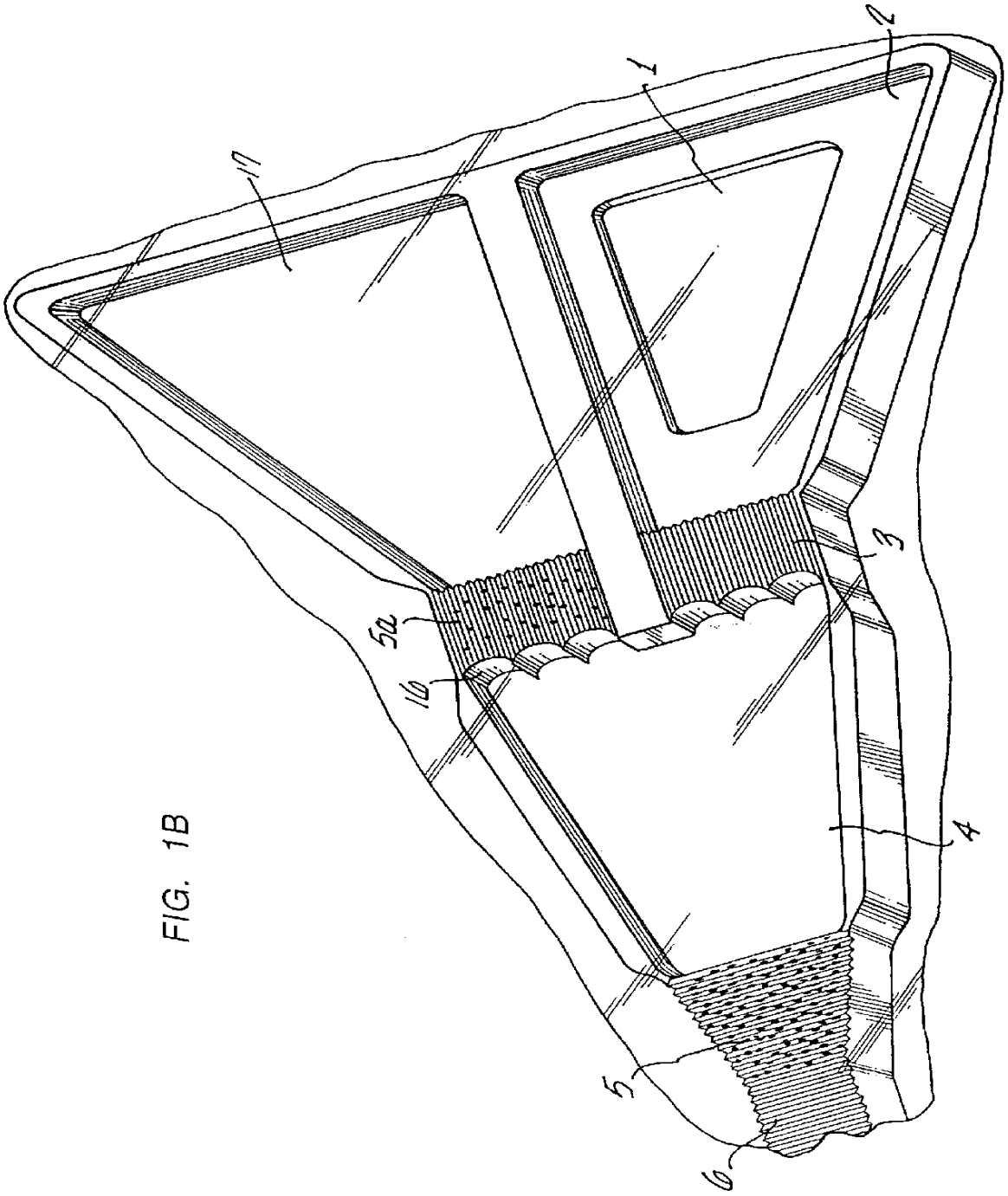


FIG. 1B

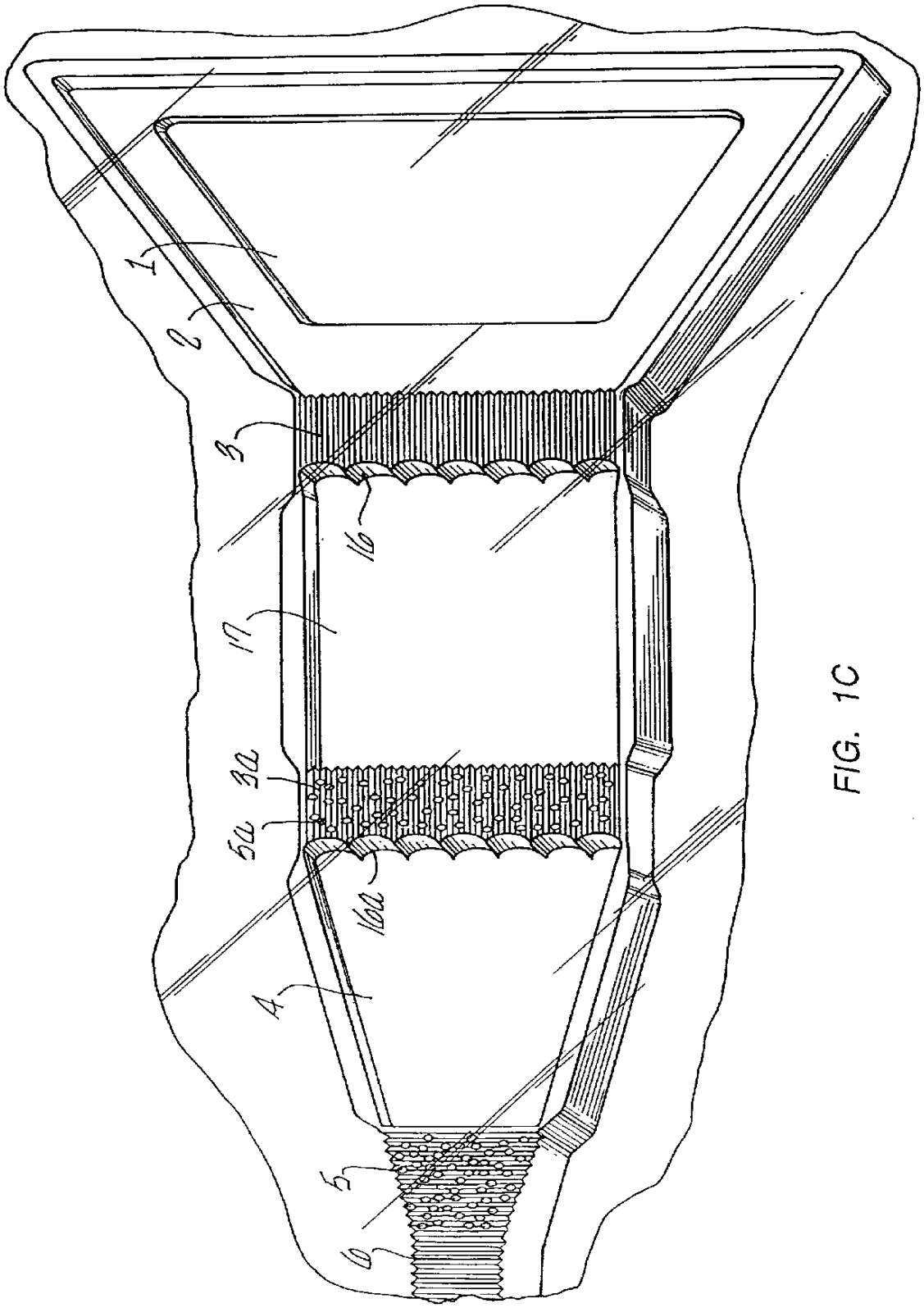


FIG. 1C

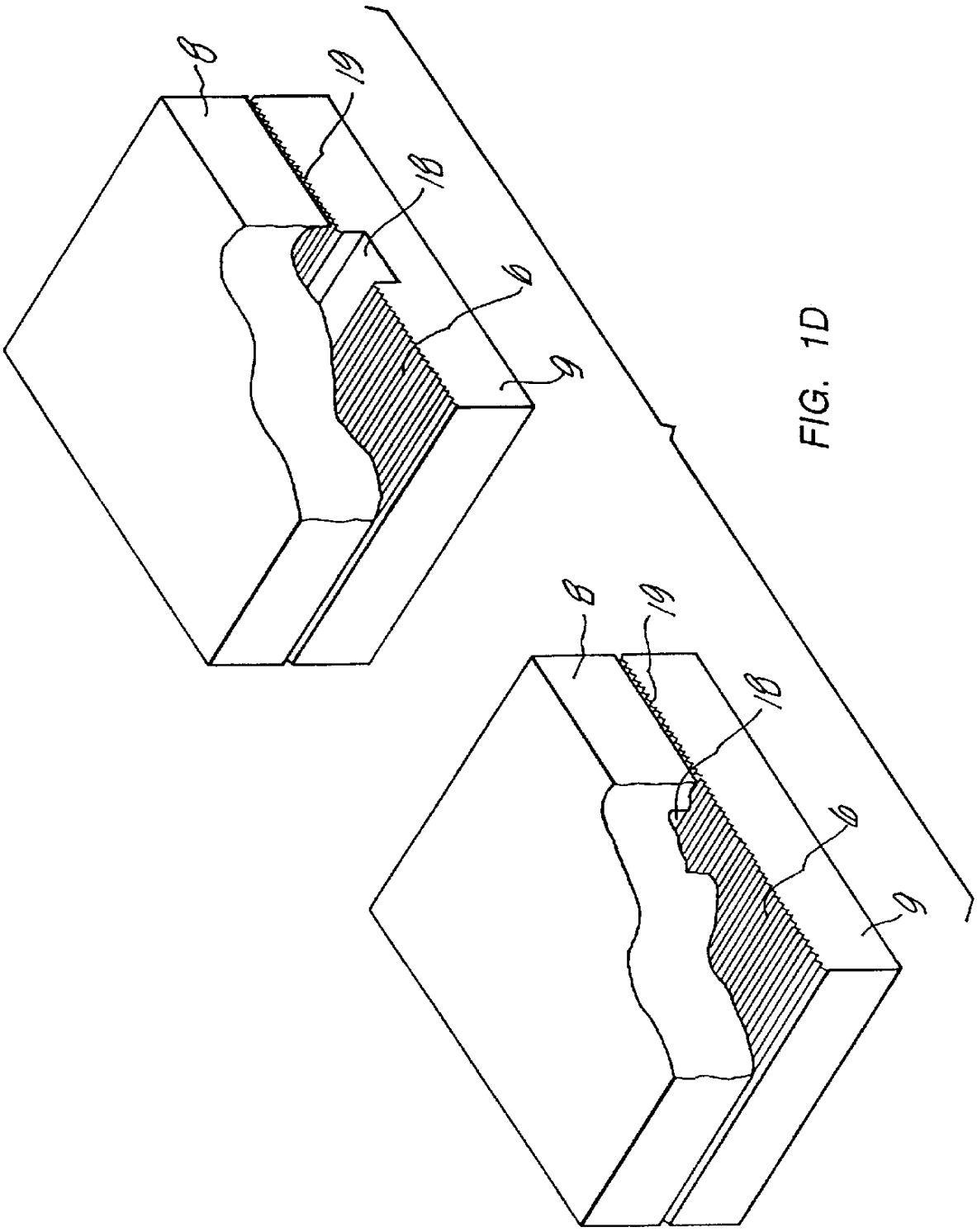


FIG. 1D

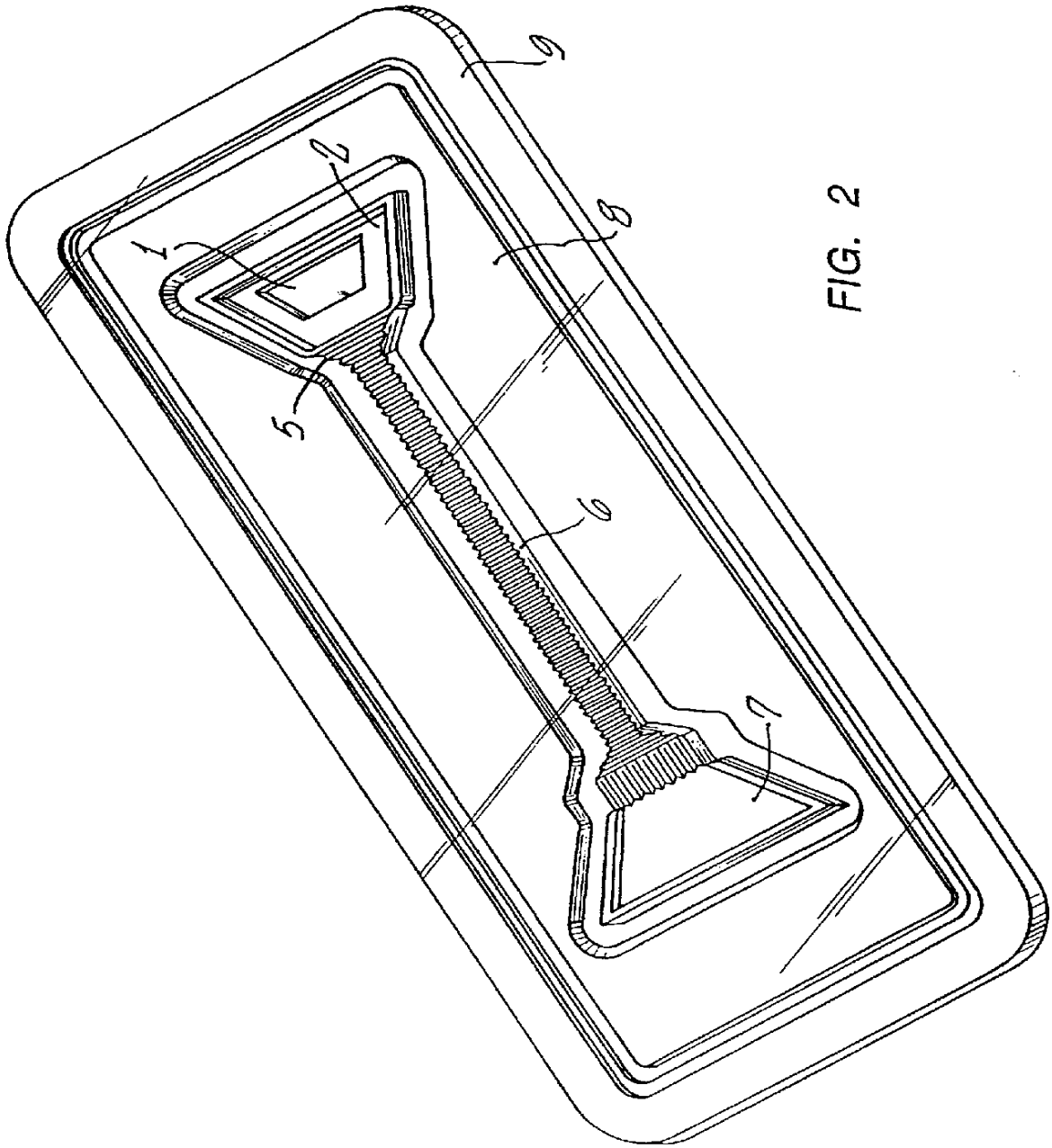


FIG. 2

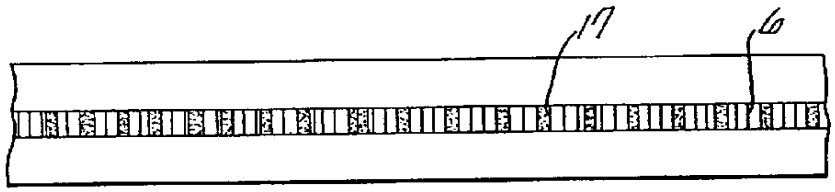


FIG. 3A

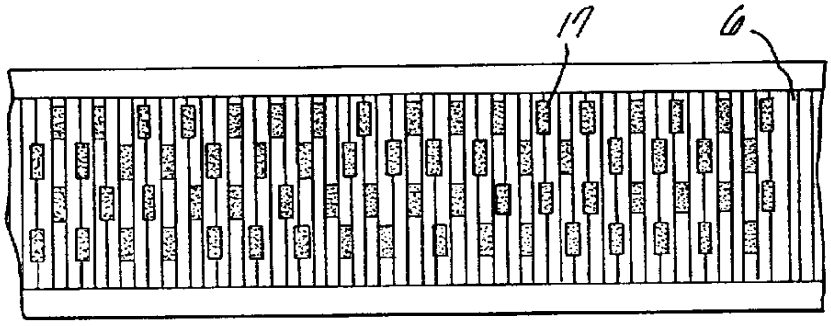


FIG. 3B

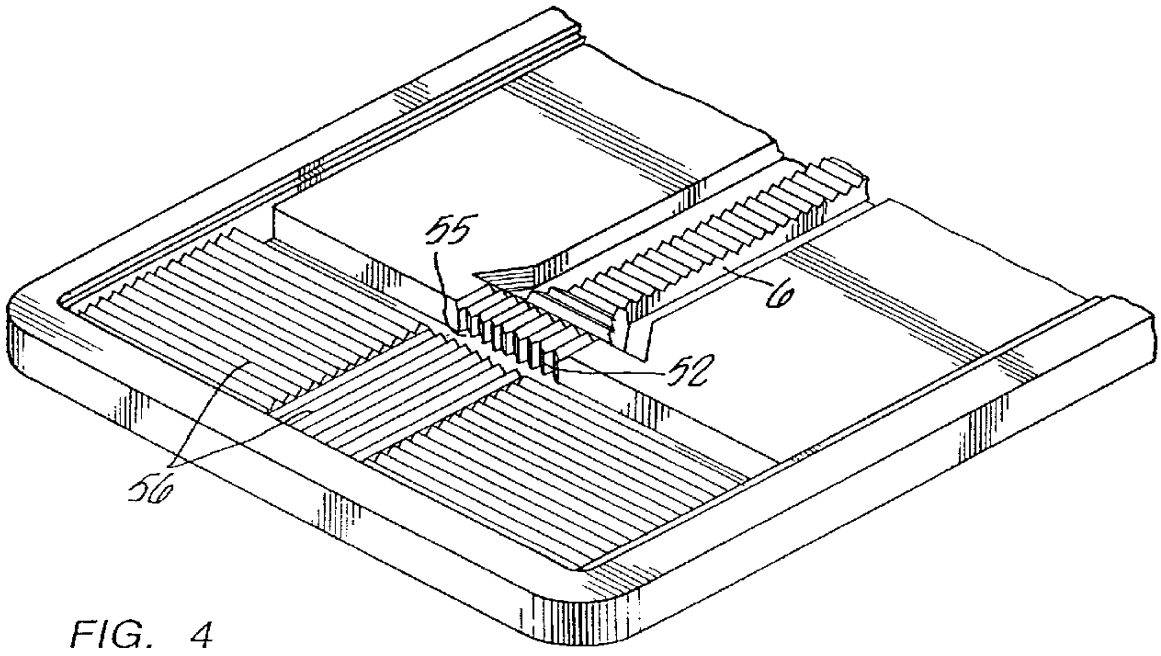


FIG. 4

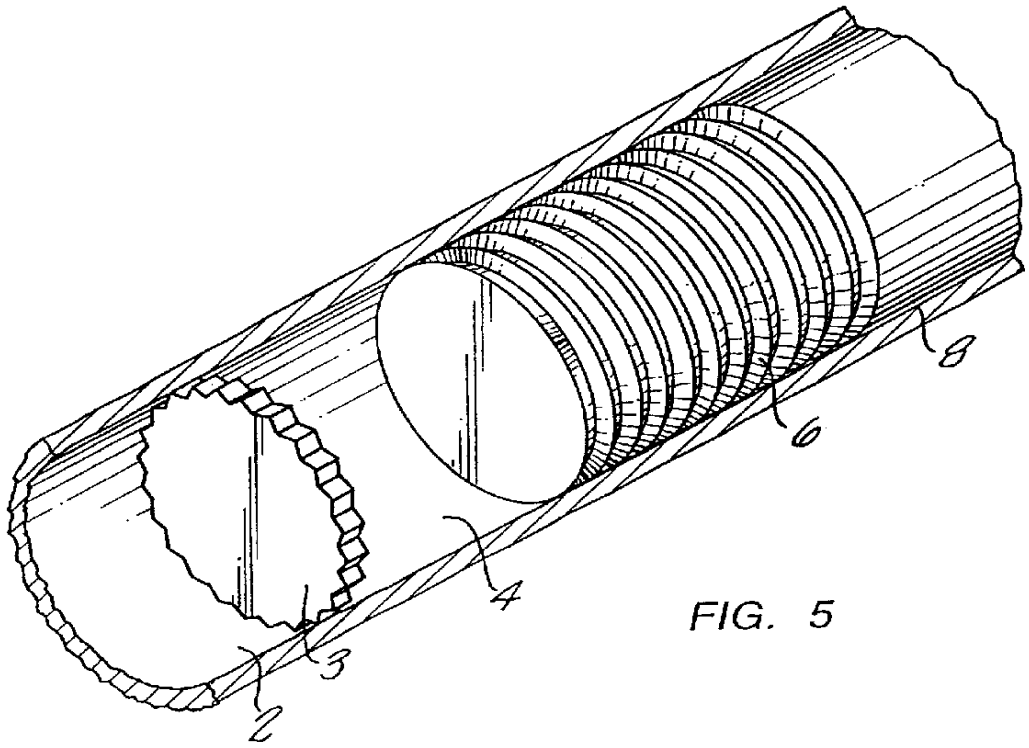


FIG. 5

FIG. 6A

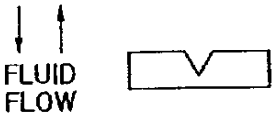


FIG. 6B

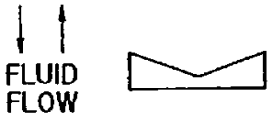


FIG. 6C

FIG. 6D

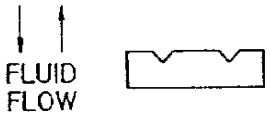
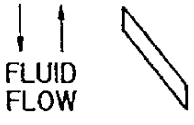


FIG. 6E

FIG. 6F



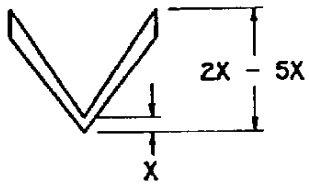


FIG. 7A

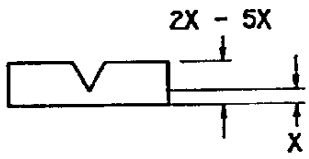


FIG. 7B

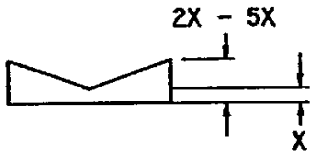


FIG. 7C

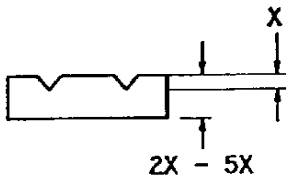


FIG. 7D

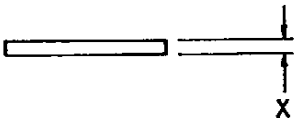


FIG. 7E

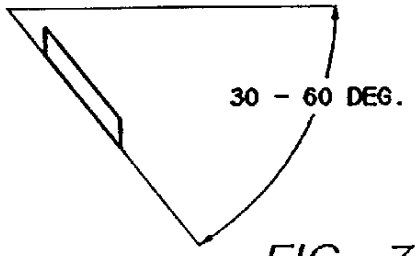


FIG. 7F

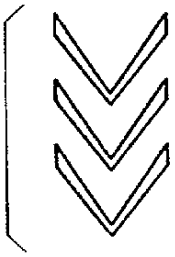


FIG. 8A

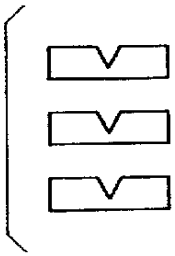


FIG. 8B

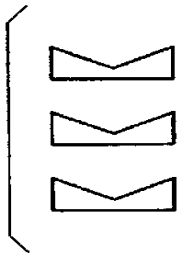


FIG. 8C

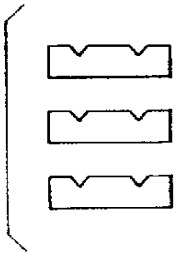


FIG. 8D

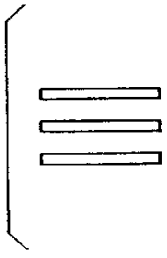


FIG. 8E

FIG. 8F

DIAGNOSTIC DEVICES AND APPARATUS FOR THE CONTROLLED MOVEMENT OF REAGENTS WITHOUT MEMBRANES

This application is a divisional of, app. Ser. No. 08/065,528, filed May 19, 1993, now abandoned which was a continuation-in-part of app. Ser. No. 07/887,526 filed May 21, 1992 now U.S. Pat. No. 5,458,852. Priority is claimed from each of these applications.

FIELD OF THE INVENTION

This invention relates to devices for conducting assays, including qualitative, semi-quantitative and quantitative determinations of one or more analytes in a single test format. Unlike assay devices of the prior art, the inventive assay devices described herein do not involve the use of bibulous materials, such as papers or membranes. The inventive devices of the present invention rely on the use of defined surfaces, including grooved surfaces, and capillarity alone or in various combinations to move the test reagents. The inventive devices described herein provide means for the controlled, timed movement of reagents within the device and do not require precise pipetting steps. The concepts and devices of the present invention are especially useful in the performance of immunoassays of environmental and industrial fluids, such as water, and biological fluids and products, such as urine, blood, serum, plasma, spinal and amniotic fluids and the like.

BACKGROUND OF THE INVENTION

Over the years, numerous simplified test systems have been designed to rapidly detect the presence of a target ligand of interest in biological, environmental and industrial fluids. In one of their simplest forms, these assay systems and devices usually involve the combination of a test reagent which is capable of reacting with the target ligand to give a visual response and an absorbent paper or membrane through which the test reagents flow. Paper products, glass fibers and nylon are commonly used for the absorbent materials of the devices. In certain cases, the portion of the absorbent member containing the test reagents is brought into contact, either physically or through capillarity, with the sample containing the target ligand. The contact may be accomplished in a variety of ways. Most commonly, an aqueous sample is allowed to traverse a porous or absorbent member, such as porous polyethylene or polypropylene or membranes by capillarity through the portion of theorous or absorbent member containing the test reagents. In other cases, the test reagents are pre-mixed outside the test device and then added to the absorbent member of the device to ultimately generate a signal.

Commercially available diagnostic products employ a concentrating zone methodology. In these products, such as ICON® (Hybritech Incorporated), TESTPACK™ (Abbott Laboratories) or ACCULEVEL® (Syva Corporation), the device contains an immunosorbing or capture zone within a porous member to which a member of a specific binding pair is immobilized. The surface of the porous member also may be treated to contain one or more elements of a signal development system. In these devices, there is a liquid absorbing zone which serves to draw liquid through the immunosorbing zone, to absorb liquid sample and reagents and to control the rate at which the liquid is drawn through the immunosorbing zone. The liquid absorbing zone is either an additional volume of the porous member outside of the immunosorbing zone or an absorbent material in capillary

communication with the immunosorbing zone. Many commercially available devices and assay systems also involve a wash step in which the immunosorbing zone is washed free of non-specifically bound signal generator so that the presence or amount of target ligand in the sample can be determined by examining the porous member for a signal at the appropriate zone.

The devices described herein do not use bibulous or porous materials, such as membranes and the like as substrates for the immobilization of reagents or to control the flow of the reagents through the device. A disadvantage of, for example, membranes in diagnostic devices is that on both microscopic and macroscopic scales the production of membranes is not easily reproducible. This can result in diagnostic devices which have differential properties of non-specific binding and flow characteristics. The time gates of this invention can, however, be embedded in membranes or used in devices with membanes. Membranes are very susceptible to non-specific binding which can raise the sensitivity limit of the assay. In the case of immunochromatographic assay formats such as those described in U.S. Pat. Nos. 4,879,215, 4,945,205 and 4,960,691, the use of membranes as the diagnostic element requires an even flow of reagents through the membrane. The problem of uneven flow of assay reagents in immunochromatographic assays has been addressed in U.S. Pat. Nos. 4,756,828, 4,757,004 and 4,883,688, incorporated herein by reference. These patents teach that modifying the longitudinal edge of the bibulous material controls the shape of the advancing front. The devices of the current invention circumvent these membrane associated problems by the use of defined surfaces, including grooved surfaces, capillarity, time gates, novel capillary means, including channels and novel fluid flow control means alone or in various combinations, all of which are constructed from non-absorbent materials. In a preferred mode of this invention, the capillary channel of the diagnostic element is composed of grooves which are perpendicular to the flow of the assay reagents. The manufacture of grooved surfaces can be accomplished by injection molding and can be sufficiently reproducible to provide control of the flow of reagents through the device.

In addition to the limitations of the assay devices and systems of the prior art, including the limitations of using absorbent membranes as carriers for sample and reagents, assay devices generally involve numerous steps, including critical pipetting steps which must be performed by relatively skilled users in laboratory settings. Accordingly, there is a need for one step assay devices and systems, which, in addition to controlling the flow of reagents in the device, control the timing of the flow of reagents at specific areas in the device. In addition, there is a need for assay devices which do not require critical pipetting steps but still perform semiquantitative and quantitative determinations. The inventive devices and methods of this invention satisfy these needs and others by introducing devices which do not require precise pipetting of sample, which do not use absorbent members, which include novel elements called time gates for the controlled movement of reagents in the device and which are capable of providing quantitative assays.

Definitions

In interpreting the claims and specification, the following terms shall have the meanings set forth below.

Target ligand—The binding partner to one or more receptors.

Ligand—Binding partner to a ligand receptor.

Ligand Analogue—A chemical derivative of the target ligand which may be attached either covalently or non-

covalently to other species, for example, to the signal development element. Ligand analogue and target ligand may be the same and both are capable of binding to the receptor.

Ligand Analogue conjugate—A conjugate of a ligand analogue and a signal development element;

Signal Development Phase—The phase containing the materials involving the signal development element to develop signal, e.g., an enzyme substrate solution.

Receptor—Chemical or biochemical species capable of reacting with or binding to target ligand, typically an antibody, a binding fragment, a complementary nucleotide sequence or a chelate, but which may be a ligand if the assay is designed to detect a target ligand which is a receptor. Receptors may also include enzymes or chemical reagents that specifically react with the target ligand.

Ligand Receptor Conjugate—A conjugate of a ligand receptor and a signal development element.

Signal Development Element—The element which directly or indirectly causes a visually or instrumentally detectable signal as a result of the assay process. Receptors and ligand analogues may be bound, either covalently or noncovalently to the signal development element to form a conjugate. The element of the ligand analogue conjugate or the receptor conjugate which, in conjunction with the signal development phase, develops the detectable signal, e.g., an enzyme.

Reaction Mixture—The mixture of sample suspected of containing target ligand and the reagents for determining the presence or amount of target ligand in the sample, for example, the ligand analogue conjugate or the receptor conjugate. As used herein the Reaction Mixture may comprise a proteinaceous component which may be the target, a component of the sample or additive (e.g., serum albumin, gelatin, milk proteins).

Ligand Complement—A specialized ligand used in labelling ligand analogue conjugates, receptors, ligand analogue constructs or signal development elements.

Ligand Complement Receptor—A receptor for ligand complement.

Ligand Analogue-Ligand Complement Conjugate—A conjugate composed of a ligand analogue, a ligand complement and a signal development element.

Capture Efficiency—The binding efficiency of the component or components in the reaction mixture, such as the ligand analogue conjugate or the receptor conjugate, to the capture zone of the diagnostic element.

Capture Zone—The area on the diagnostic element which binds at least one component of the reaction mixture, such as the ligand analogue conjugate or the receptor conjugate.

Capillarity—The state of being capillary or the exhibition of capillary action. Capillarity can be affected by the solid surface or the liquid surface or both.

Biosensor—Any electrochemical, optical, electrooptical or acoustic/mechanical device which is used to measure the presence or amount of target ligands. For example, electrochemical biosensors utilize potentiometric and amperometric measurements, optical biosensors utilize absorbance, fluorescence, luminescence and evanescent waves. Acoustic/mechanical biosensors utilize piezoelectric crystal resonance, surface acoustic waves, field-effect transistors, chemical field-effect transistors and enzyme field-effect transistors. Biosensors can also detect changes in the physical properties of solutions in which receptor binding events take place. For example, biosensors may detect changes in the degree of agglutination of latex particles upon binding antigen or they may detect changes in the viscosity of solutions in response to receptor binding events.

DESCRIPTION OF THE DRAWINGS

FIG. **1** is a partially schematic, top perspective view of a device in accordance with the present invention.

FIG. **1***a* is a partially schematic, perspective exploded view of the device showing the detail in the area of the sample addition reservoir, the sample-reaction barrier, the reaction chamber, the time gate and the beginning of the diagnostic element.

FIG. **1***b* is a partially schematic, perspective exploded view of the device showing the detail in the area of the optional reagent reservoir, the sample addition reservoir, the sample-reaction barrier, the reaction chamber, the time gate and the beginning of the diagnostic element.

FIG. **1***c* is a partially schematic, perspective exploded view of the device showing the detail in the area of the optional reagent reservoir in fluid contact with the sample addition reservoir and the reaction chamber.

FIG. **1***d* is a partially schematic. perspective cutaway view of the flow control means.

FIG. **2** is a partially schematic, perspective view of a second device in accordance with this present invention, which may be used to add pre-mixed reaction mixtures.

FIGS. **3**(*a* and *b*) are a partially schematic top view of the diagnostic element showing some potential placements of capture zones.

FIG. **4** is a partially schematic, perspective view of a used reagent reservoir.

FIG. **5** is a partially schematic view of embodiments of these devices which are columnar or have curved opposing surfaces.

FIGS. **6**(*a–f*) are a top view of time gates.

FIGS. **7**(*a–f*) show typical dimensions for a preferred time gate.

FIGS. **8**(*a–f*) are a top view of sequential time gates.

SUMMARY OF THE INVENTION

The assay devices, assay systems and device components of this invention comprise at least two opposing surfaces disposed a capillary distance apart, at least one of which is capable of immobilizing at least one target ligand or a conjugate in an amount related to the presence or amount of target ligand in the sample from a fluid sample in a zone for controlled fluid movement to, through or away the zone. The inventive device components may be incorporated into conventional assay devices with membranes or may be used in the inventive membrane-less devices herein described and claimed. These components include flow control elements, measurement elements, time gates, elements for the elimination of pipetting steps, and generally, elements for the controlled flow, timing, delivery, incubation, separation, washing and other steps of the assay process.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to diagnostic testing devices for determining the presence or amount of at least one target ligand. FIG. **1** shows a preferred embodiment of a device **10** according to the invention. Generally, the devices of the invention have thicknesses of about 2 mm to 15 mm, lengths of about 3 cm to 10 cm and widths of about 1 cm to 4 cm. The dimensions may be adjusted depending on the particular purpose of the assay. One device of this invention, as depicted in FIG. **1**, generally illustrates some features of the inventive devices and portions of devices

herein disclosed and claimed. The device **10** comprises various elements, a sample addition zone **1**, a sample addition reservoir **2**, a sample reaction barrier **3**, a reaction chamber **4**, a time gate **5**, a diagnostic element **6**, and a used reagent reservoir **7**. The devices are comprised of capillary channels which are formed when a top member **8** is placed on the bottom member **9** a capillary distance apart and which move the reagents and sample throughout the device. The top and bottom members may be married, the various chambers sealed and the capillaries formed by a number of techniques, including but not limited to, gluing, welding by ultrasound, riveting and the like. The elements of the device can be used in various combinations with the diagnostic element **6** to achieve a variety of desired functions. As one skilled in the art will recognize these elements may be combined to perform one-step or multistep assays. The devices **10** may also be used in the formation of reaction mixtures for the assay process. The device **20** in FIG. **2** may be used to add pre-mixed reaction mixtures for the generation of signal which relates to the presence or amount of the target ligand.

An optional reagent chamber **17** may be incorporated into device **10** or **20** as depicted in FIG. **1***b* and FIG. **1***c*. The devices **10** and **20** may also be used with an optional fluid control means **18** as shown in FIG. **1***d*.

Features include, but are not limited to: 1) diagnostic elements which are not comprised of bibulous materials, such as membranes, 2) means to control the volume of sample or reaction mixture, 3) time gates, 4) novel capillary means, termed fingers herein and 5) novel flow control means, sometimes referred to as a "gap" herein and 6) used reagent reservoir which prevents backward flow of reagents. Those of skill in the art will appreciate that these elements are separately novel and nonobvious, and may be incorporated into diagnostic devices in various combinations and may be used with other elements known to those skilled in the art to achieve novel and nonobvious diagnostic test devices and heretofore unrealized benefits.

Each of the elements of devices **10** and **20** will be described separately, then representative descriptions of the devices of this invention will follow.

Sample Addition Zone

Referring to FIGS. **1** and **2**, the sample addition zone **1** of the devices **10** and **20** is the area where sample is introduced to the device. The sample addition zone **1** can be a port of various configurations, that is, round, oblong, square and the like or the zone can be a trough in the device.

Sample Addition Reservoir

Referring to FIGS. 1 and **2**, the sample addition reservoir **2** is an element of the device which receives the sample. Referring now to FIG. **1**, the volume of the sample addition reservoir **2** should be at least the volume of the reaction chamber **4** or greater. The sample addition reservoir **2** can be a capillary space or it can be an open trough. In addition, a filter element can be placed in or on the sample addition reservoir **2** to filter particulates from the sample or to filter blood cells from blood so that plasma can further travel through the device. In a preferred embodiment, the volume or capacity of the sample addition reservoir **2** is 1 to 5 times the volume of the reaction chamber **4**. In general, one selects a volume or capacity of this reservoir **2** such that if the excess sample is used to wash the diagnostic element **6** then enough volume of sample is needed to thoroughly remove any unbound reagents from the diagnostic element **6** arising from the assay process. This reservoir **2** may also contain certain dried reagents which are used in the assay process. For example, a surfactant can be dried in this reservoir **2** which dissolves when sample is added. The surfactant in the sample would aid in the movement of the sample and reaction mixture through the device by lowering the surface tension of the liquid. The sample addition reservoir **2** is generally in direct fluid contact with the sample-reaction barrier **3** (FIG. **1**) or the diagnostic element **6** (FIG. **2**).

Sample-Reaction Barrier

As depicted in FIG. **1**, the sample-reaction barrier **3** separates the sample in the sample addition reservoir **2** from the reaction mixture in the reaction chamber **4**. The sample-reaction barrier is desired because it provides the device with the capability of forming a precise reaction mixture volume. A precise volume of the reaction mixture is generally necessary for assays in which semi-quantitative or quantitative results are desired. Thus, a precise pipetting step of the sample to the device is not required because the sample reaction barrier forms a reaction chamber of precise volume into which the sample is capable of flowing. The sample reaction barrier **3** is desired because the reactions which take place in the reaction chamber **4** should preferably be separated from the excess sample in the sample addition reservoir **2**. The sample reaction barrier **3** comprises a narrow capillary, generally ranging from about 0.01 mm to 0.2 mm and the surfaces of the capillary can be smooth or hive a single groove or a series of grooves which are parallel or perpendicular to the flow of sample. In a preferred embodiment of the sample reaction barrier **3**, now referring to FIG. **1***a*, grooves **12**, parallel to the flow of sample, are incorporated onto one surface of the device a capillary distance, for example, 0.02 mm to 0.1 mm, from the other surface. The volume of sample which fills the sample-reaction barrier **3** (FIG. **1***a*) should be kept to a minimum, from about 0.01% to 10% of the reaction chamber **4** volume so that the reagents of the reaction chamber **4** do not significantly diffuse back into the sample in the sample addition reservoir **2**. That is, the diffusion of the reaction mixture back into the excess sample should be kept to a minimum so that the chemical or biochemical reactions occurring in the reaction mixture are not substantially influenced by the excess sample in the sample addition reservoir **2**. Groove depths can range from about 0.01 mm to 0.5 mm and preferably from about 0.05 mm to 0.2 mm. When more than one groove is used for this element, the number of grooves in this element is typically between 10 and 500 grooves per cm and preferably from about 20 to 200 grooves per cm. Sample from the sample addition reservoir **2** flows over the grooves **12** by capillary action and then into the reaction chamber **4**. In a further preferred embodiment, grooves, hereafter termed "fingers" **16**, are situated in the wall of the reaction chamber **4** in fluid contact with the grooves **12** or capillary space of the sample-reaction barrier **3**. These fingers **16** are typically 0.5 mm to 2 mm wide, preferably 1 mm to 1.5 mm wide and typically 0.1 mm to 1.5 mm in depth, preferably about 0.2 to 1 mm in depth. The fingers **16** in the wall of the reaction chamber **4** aid in the capillary flow of the sample into the reaction chamber **4**. That is, the fingers allow fluid to move from a capillary where the capillarity is relatively high to a capillary where the capillarity is lower. Thus, the capillary at the sample-reaction barrier is generally more narrow and has a greater capillarity than the capillary or space of the reaction chamber. This difference in capillarity can cause the flow of sample or fluid in the device to stop in the sample-reaction barrier capillary. Presumably, the fingers break the surface tension of the fluid at the interface of the two capillaries or spaces and thereby cause the fluid to move into a capillary or space of lower capillarity. One can appreciate that the

utility of fingers can be extended to any part of the device where fluid must flow from high capillarity to low capillarity. In practice, this is usually when the direction of fluid flow is from a narrow capillary (higher capillarity)to a wider capillary (lower capillarity). The top surface of the sample reaction barrier may also be used to immobilize reagents used in the assay process such that the sample flows over the sample reaction barrier, dissolves the reagents and moves into the reaction chamber. The movement of the sample and reagents into the reaction chamber may act as a mixing means.

Reaction Chamber

Referring to FIG. **1**, the sample moves into the reaction chamber **4** from the sample-reaction barrier **3**. The reagents of the device **10** are preferably placed in the reaction chamber **4**, for example, as dried or lyophilized powders, such that when the sample enters the reaction chamber **4** the reagents quickly reconstitute. The volume of the reaction chamber **4** is the volume of sample which defines the reaction mixture. The reaction chamber may be sealed on **2** sides, for example, by ultrasonic welding of the top and bottom members. Thus, delivery of the sample to the device **10** at the sample addition zone **1** does not require a precise pipetting step to define the volume of the reaction mixture. Mixing features which mix the reaction mixture can also be incorporated in conjunction with the reaction chamber element **4**, such as those described in U.S. pat. appl. Ser. No. 711,621 filed Jun. 5, 1991, hereby incorporated by reference. The sample fills the reaction chamber **4** because of capillary forces and also, potentially, because of the hydrostatic pressure exerted by the sample in the sample addition reservoir **2**. The floor of the reaction chamber **4** may be smooth or comprised of a grooved surface to aid in the movement of the sample into the reaction chamber **4**. The volume of the reaction chamber **4**, and thereby the reaction mixture, may be any volume which accommodates the reagents and which provides the desired sensitivity of the assay. The shape of the reaction chamber **4** should be such that the movement of the reaction mixture from the reaction chamber **4** is not turbulent and eddies are not formed as a result of the movement out of the reaction chamber **4**. A preferred shape of the reaction chamber **4** is shown in FIG. **1**. The depth of the reaction chamber **4** should be commensurate with the width of the chamber to accommodate the desired reaction mixture volume. The depth of the reaction chamber can range from about 0.05 mm to 10 mm and preferably from 0.1 mm to 0.6 mm. To accommodate a particular volume of the reaction chamber, the length and width of the reaction chamber should be adjusted and the depth maintained as narrow as is practical. The reaction chamber **4** is in direct fluid contact with the sample-reaction barrier **3** and the diagnostic element **6** or time gate **5**. In addition, the reaction chamber **4** may also be in direct fluid contact with an optional reagent reservoir **17** as shown in FIGS. **1***b* and **1***c*.

A preferred embodiment of the reaction chamber utilizes a ramp which extends from the bottom of the reaction chamber to the surface of the diagnostic element. The ramp minimizes or prevents mixing and eddie formation of the reaction mixture with the sample at the interface of the reaction chamber and the diagnostic element as the fluid moves through the device. Thus, the ramp allows a smooth transition of the fluid out of the reaction chamber and onto the diagnostic element. The length of the ramp should be optimized for each depth of the reaction chamber, but generally, the ramp is at an angle of between 25 and 45 degrees relative to the floor of the reaction chamber.

Time Gate

Referring to FIG. **1***a*, the time gate **5** holds the reaction mixture in the reaction chamber **4** for a given period of time. The concept of the time gate is that a predominantly aqueous solution cannot pass through a sufficiently hydrophobic zone until the hydrophobic zone is made sufficiently hydrophilic. Furthermore, the hydrophobic zone is made hydrophilic through the binding of a component in the aqueous solution to the hydrophobic zone. The sufficiently hydrophobic zone is generally in a capillary space. The driving force for fluid movement over or through the time gate may be either the capillarity of the space or hydrostatic pressure exerted by the sample or a combination of both of these forces. The amount of time which is required to hold the reaction mixture in the reaction chamber **4** is relative to the assay process such that the reactions which occur in the reaction chamber **4** as a result of the assay process will reflect the presence or amount of target ligand in the sample. Thus, the time gate **5** delays the flow of the reaction mixture onto the diagnostic element **6**. The time gate **5** delays the flow of the reaction mixture by the principle that a hydrophilic liquid, such as an aqueous solution or one which has a dielectric constant of at least 40, cannot move past a sufficiently hydrophobic barrier in a capillary channel. In designing and building a time gate, one can begin with a hydrophobic surface, such as are found on native plastics and elastomers (polyethylene, polypropylene, polystyrene, polyacrylates, silicon elastomers and the like) or silicon chip surfaces or metal surfaces, either smooth, grooved or textured and a capillary is formed by an opposing surface which can be hydrophobic or hydrophilic in nature and smooth, grooved or textured. The hydrophobic surface(s) in the capillary have. a microscopic surface area onto which can bind components which are generally soluble in a predominantly aqueous solution. The hydrophilic character and the concentration of the component(s) in the reaction mixture and the overall surface area of the time gate affects the mechanics of the time gate. The amount of time for which the time gate **5** holds the reaction mixture is related to the rate of binding of a component(s) from the reaction mixture to the hydrophobic barrier. The binding of the component(s) from the reaction mixture changes the hydrophobic barrier to a zone which is sufficiently hydrophilic over which or through which the reaction mixture can flow. Creating the sufficiently hydrophilic surface then allows the fluid to flow as if the time gate had not been in the device. Thus, fluid flow through the remainder of the device is not affected once the time gate has been made hydrophilic. Other devices described which incorporate fluid delay means, for example, in U.S Pat. Nos. 4,426,451 and 4,963,498, hereby incorporated by reference only, require an external manipulation of the device to start fluid flow or utilize capillary constrictions to slow fluid flow. In this latter case, the capillary constriction used to delay fluid flow will affect the fluid flow through the remainder of the device.

In a preferred embodiment, for example, the time gate can be composed of latex particles **15** (FIG. **1***a*, not drawn to scale), such as polystyrene latexes with diameters of between about 0.01 μm and 10 μm or hydrophobic polymers, such as polypropylene, polyethylene, polyesters and the like, which are introduced onto the device in the appropriate zone where the reaction mixture must travel. In another preferred embodiment, the time gate can be created by application of a hydrophobic chemical, such as an ink or a long chain fatty acid, or a hydrophobic decal to the desired zone. The hydrophobic chemical or decal is generally not soluble or is poorly soluble in the reaction mixture. In yet another pre-

ferred embodiment, the time gate can also be formed by changing a hydrophilic surface to a hydrophobic surface. For example, hydrophobic surfaces made hydrophilic by plasma treatment can be converted back to a hydrophobic surface by the application of solvents, ultraviolet light or heat and the like. These treatments can act to change the molecular structure of the hydrophilic, plasma modified surface back to a hydrophobic form.

The component(s) in the reaction mixture which bind to the hydrophobic zone may be various proteins, polypeptides, polymers or detergents. A preferred protein is bovine serum albumin. The time delay provided by the time gate **5** depends on the concentration of the component(s) in the reaction mixture, for example, bovine serum albumin, which binds to the hydrophobic zone, for example, the surface area provided by the latex particles **15**. Another preferred embodiment of the time gate **5** utilizes polyelectrolytes which are hydrophobic and which become hydrophilic by exposure to the buffering capacity of the reaction mixture. The time gate **5** would be comprised of, for example, polyacrylic acid, which in its protonated form it is hydrophobic. The reaction mixture, if buffered above the pK_a of the polyacrylic acid, would deprotonate the acid groups and form the hydrophilic salt of the polymer. In this case, the time delay is related to the mass of polyelectrolyte and the pH and the buffering capacity of the reaction mixture.

The geometry or shape of the time gate can influence the area of the time gate that the fluid will pass over or through. That is, the time gate can be designed to direct the flow of liquid through a specific area of the time gate. By directing the fluid to flow through a defined area of the time gate the reproducibility of the time delay is improved. FIG. **6** shows representative geometries of time gates. For example, as shown in FIG. **6**, time gates a–d, the time gates have V-shapes incorporated into their design, and more specifically, the length of the time gate (defined as the distance the fluid must cross over or through in order to pass the time gate) is less at the tip of the V than in the body of the time gate. Thus, in a preferred mode, the fluid will cross over or pass through the time gate where the length is shortest thereby directing fluid flow through the time gate in a consistent manner. In general, the directionality of fluid flow over or through the time gates is represented by opposing arrows in FIG. **6**. In a preferred embodiment, the orientation of the time gates b, c and d of FIG. **6** are such that the fluid touches the flat portion of the time gate first rather than the V shape. In other words, the preferred direction of flow for the time gates b, c and d of FIG. **6** is represented by the up arrow. In cases where the time gate is simply a line, for example as seen in FIG. **6**, time gate e and f, the path of fluid flow over or through the time gate can occur at any point on the time gate. Thus, the time gates which have geometries directing the fluid flow over or through a consistent area of the time gate are preferred. For example, time gates with lengths ranging from about 1.3 mm to 0.13 mm achieve delay times of approximately 0.3 min to 5.5 min, respectively, when the distance between surfaces is about 0.018 mm. When the time gate is V-shaped, the length of the time gate at the tip of the V has dimensions smaller than the length of the time gate at the remaining portion of the V; that is, the arms of the V should have a length roughly 2 to 5 times the length of the V tip, as for example, FIG. **7**, time gate a, illustrates. FIG. **7**, time gate b, shows that only a small area of the time gate is crossed over or through at the tip of the V as compared with the remainder of the time gate. The time gate should span the width of the capillary or space so that the entire fluid front comes in contact with the time gate. If the time gate was not as wide as, for example, the diagnostic element, then the fluid front would go around the time gate. Thus, the time gate should "seal" the fluid in the space during the delay period.

Referring to FIG. **1**, one skilled in the art can recognize that each device **10** could incorporate one or more time gates to achieve the desired function of the device. FIG. **8** shows some examples of the sequential placement of several time gates of FIG. **6**. For example, as discussed in the next section, Optional Reagent Chambers, if a sequential addition immunoassay was to be performed by the device then 2 time gates would allow 2 sequential incubation steps to be performed by the device prior to the movement of the reaction mixture to the diagnostic element. In another example, if an incubation of the reaction mixture on the capture zone or zones of the diagnostic element(s) **6** was required then a time gate(s) would be placed immediately behind the capture zone or zones. This use of the time gate may arise in cases where poor efficiency of binding of the component in the reaction mixture to the capture zone of the diagnostic element would prevail.

Another application of the time gate involves the placement of a time gate on a surface which is not part of a capillary space. For example, the time gate can be placed on a hydrophilic surface, which alone without a capillary space will allow liquids to move. This is generally the case when a substantial volume of liquid is placed on a surface and it spreads because of surface tension and because of the hydrostatic pressure of the liquid pushing the meniscus outwardly. The time gate then would function to delay the advance of the fluid front because the hydrostatic nature of the surface of the time gate would stop the movement of liquid. As the meniscus of the advancing liquid touches the time gate, the component or components in the liquid binds to the time gate to create a sufficiently hydrophilic surface for a continued advance of the liquid on the surface.

Yet another embodiment of the time gate involves the positioning of a time gate prior to a membrane which is used to capture a conjugate or receptor. In yet another embodiment of the time gate, the time gate can be composed of hydrophobic surfaces in a membrane. In those cases, the hydrophobic membrane is positioned prior to the portion of membrane which captures the conjugate or receptor and may be positioned after a reaction chamber or a portion of membrane where reagents of the assay are placed or embedded and where the reagents incubate for a defined period of time. The time gate in the membrane can be formed by application of raw latex particles in the membrane at an appropriate solids concentration ranging from about 0.01% to 10%. The size of the latex particles should be slightly less than the pore size of the membrane so that the latex becomes imbedded within the membrane. The density of latex within the membrane at the time gate should be uniform so that the reaction mixture does not circumvent the time gate. For example, the latex size used to create a time gate for a membrane with a pore size of 1 μm can range between 0.05 and 0.2 μm. Since the distribution of pore sizes in membranes varies widely, the actual size of latex used must be arrived at by experimentation. The hydrophobic nature of the membrane used for the time gate can also be formed by plasma treatment or by treatment of the membrane with hydrophobic chemicals or polymers that adsorb to the membrane. One skilled in the art can appreciate that the teachings described herein of the inventive features of the time gate can be utilized to design time gates in a variety of diagnostic devices which utilize membranes. That is, devices described, for example, in U.S. Pat. Nos. 4,435,504, 4,727,

019, 4,857,453, 4,877,586 and 4,916,056, and . . . hereby incorporated by reference, can incorporate a time gate, for example, prior to the membrane or in the membrane which captures the conjugate or receptor.

Optional Reagent Chambers

Referring to FIGS. 1*b* and 1*c*, the optional reagent chamber **17** is useful for the introduction of reagents into the assay process. In general, the optional reagent chamber **17** may be in direct fluid contact with the sample addition reservoir **2** via a sample reaction barrier **3** or a port the reaction chamber **4** or the diagnostic element **6**, via a sample reaction barrier **3** or a port. For example, FIG. 1*b* shows the optional reagent chamber **17** in direct fluid contact with the reaction chamber **4**. The flow of the introduced reagent may be controlled by a time gate **5***a* and fingers **16** can aid in the movement of reagents into the reaction chamber **4**. Referring now to FIG. 1*c*, for example, if a sequential addition immunoassay was to be performed by the device then **2** time gates **5** and **5***a* would and fingers **16** can aid in the movement of reagents into the reaction chamber **4**. Referring now to FIG. 1*c*, for example, if a sequential addition immunoassay was to be performed by the device then 2 time gates **5** and **5***a* would allow 2 sequential incubation steps to be performed in the optional reagent chamber **17** and then in the reaction chamber **4** by the device prior to the movement of the reaction mixture onto the diagnostic element **6**. That is, sample would be applied to the sample addition reservoir **2** through the sample addition zone **1** and the sample flows over the sample reaction barrier **3** and into the optional reagent chamber **17** by the aid of fingers **16** where the first set of reactions would occur. The time gate **5***a*, after the appropriate amount of time, would allow the reagents to flow over the sample reaction barrier **3***a* and into the reaction chamber **4** by the aid of fingers **16***a* where the next set of reactions would take place. After the appropriate amount of time, the time gate **5** allows the flow of reaction mixture onto the diagnostic element **6**.

Fluid Control Means

Referring to FIG. 1*d*, the optional fluid control means **18** is designed to control the flow of the reaction mixture in the device. More specifically, the optional fluid control means **18** causes the volume of the reaction mixture to flow over the capture zone of the diagnostic element **6** at a rate which allows for an optimum capture of reagents onto the capture zone. After the volume of the reaction mixture flows over the capture zone the rate of flow of the excess reagents may be increased. The differential rate of flow of the reagents in the device is achieved by designing a gap **18** between the surfaces of the capillary space **19** of the diagnostic element **6**. The size of the gap **18** is larger than the capillary space **19** of the diagnostic element **6**. The gap **18** generally follows the capture zone or the zone where the rate of flow is required to be decreased. The gap **18** in the diagnostic element **6** thus has an associated volume. The volume of the gap **18** is filled with the reaction mixture by capillary action as it moves through the device. Since the gap **18** after the capture zone is greater than the capillary space **19** of the diagnostic element **6** a drop in capillary pressure at the beginning of the gap **18** results in a decrease in the rate of flow of the reaction mixture into the gap **18** and therefore a decrease in the rate of flow of the reaction mixture over the capture zone. Varying the size of the gap **18** changes the capillarity in the gap and thus the flow of the reaction mixture over the capture zone. In the case of immunoassays requiring a wash step to remove unbound reagents from the diagnostic element **6**, it is generally desired that the rate of flow of the wash solution over the diagnostic element **6** is faster than the rate of flow of the reaction mixture over the diagnostic element **6** because this decreases the time of the assay. The shape of the gap can take many forms. As shown in FIG. 1*d*, the gap has square corners, however, the gap can be shaped as a trapezoid or triangle which would change the rate of flow of the reaction mixture while flowing into the-gap. one skilled in the art can also-appreciatethat for certain immunoassays a wash step is not required.

The control of the rate of flow of the reagents in the device can also be used to allow chemical reactions to take place in one zone of the device before the reagents move to another area of the device where the extent of reaction of the reagents is monitored or where further reaction may take place. For example, several fluid control means could be incorporated into a device for use in immunoassays where a sequential addition and incubation of reagents is necessary. That is, the sample comes in contact with the first reagents and the time for the reaction of the sample and first reagents is controlled by a first gap. When the first gap is filled with fluid, the reaction mixture continues to the second reagents at which time an additional chemical reaction can subsequently take place. The time required for completion of this second reaction can also be controlled by a second gap before further flow of the reaction mixture along the diagnostic element. Chemical and biochemical reactions also take place in the volume of the gap, for example, by immobilizing reagents in the gap.

Diagnostic Element

Referring to FIGS. **1** and **2**, the diagnostic element **6** is formed by opposing surfaces which are a capillary distance apart through which the reaction mixture flows and on which are placed one or more capture zones. The capture zones are comprised of reagents, such as receptors, or devices, such as biosensors which bind or react with one or more components from the reaction mixture. The binding of the reagents from the reaction mixture to the capture zones of the diagnostic element **6** is related to the presence or amount of target ligand in the sample. one or more receptors or biosensors can be placed on the diagnostic element **6** to measure the presence or amount of one or more target ligands. The receptors or biosensors can be placed in discrete zones on the diagnostic element **6** or they can be distributed homogeneously or heterogeneously over the surface. Receptors or other chemical reagents, for example, a receptor against the signal generator can also be immobilized on the diagnostic element **6** to verify to the user that the reagents of the reaction mixture are viable and that the reaction mixture passed through the zones of the receptors or biosensors. A single receptor or biosensor can be placed over the majority of the diagnostic element **6** such that as the reaction mixture flows through the diagnostic element **6** the components from the reaction mixture bind to the surface of the diagnostic element **6** in a chromatographic fashion. Thus, the distance which the component of the reaction mixture binds would be related to the concentration of the target ligand in the sample. The reagents, such as receptors, are immobilized on the surface of the diagnostic element **6** through covalent bonds or through adsorbtion. A preferred embodiment is to immobilize receptor coated latex particles, for example of diameters ranging from about 0.1 μm to 5 μm. In addition, particles termed "nanoparticles" can also be coated with receptor and the resulting nanoparticles can be immobilized to the diagnostic element through adsorbtion or covalent bonds. Nanoparticles are generally composed of silica, zirconia, alumina, titania, ceria, metal sols, and polystyrene and the like and the particle sizes range from about 1 nm to 100 nm. The benefit of using nanoparticles is that the surface

area of the protein coating the nanoparticle as a function of the solids content is dramatically enhanced relative to larger latex particles.

The surfaces of the diagnostic element **6** would allow the receptor coated nanoparticles or latex particles to bind to the diagnostic element **6**. In a preferred embodiment, the receptors bind to the surface of the diagnostic element through electrostatic, hydrogen bonding and/or hydrophobic interactions. Electrostatic, hydrogen bonding and hydrophobic interactions are discussed, for example, in Biochemistry 20, 3096 (1981) and Biochemistry 29, 7133 (1990). For example, the diagnostic element **6** can be treated with a plasma to generate carboxylic acid groups on the surface. The receptor coated latex particles are preferably applied to the diagnostic element **6** in a low salt solution, for example, 1–20 mM, and at a pH which is below the isoelectric point of the receptor. Thus, the negative character of the carboxylic acid groups on the diagnostic element **6** and the positive charge character of the receptor latex will result in enhanced electrostatic stabilization of the latex on the diagnostic element **6**. In another preferred embodiment, latex particles or nanoparticles, which may be coated with receptor or may compose a time gate, are entrapped on a non-absorbent surface. The microstructure of the non-absorbent surface is textured so that the particles are entrapped on the surface or in the layers of the microstructure, forming what is generally referred to as a "nanocomposite." Magnetic fields may also be used to immobilize particles which are attracted by the magnetic field. These types of surfaces, generally termed "nanostructured materials" are described, for example, in Chemical and Engineering News 70, 18–24 (1992), hereby incorporated by reference.

In an additional embodiment of the diagnostic element, now referring to FIG. **5**, the diagnostic element **6** is a cylindrical surface which may be composed of grooves. When the diagnostic element is composed of grooves, the grooves generally run perpendicular to the flow of the reaction mixture. A capillary space is formed around the diagnostic element by a round tube which is generally clear; thus, the surface of the diagnostic element and the opposing surface of the tube are a capillary distance apart. The capillary formed allows the flow of the reaction mixture over the round diagnostic element **6**. Generally, the reaction mixture would travel up against gravity or down with gravity through the cylindrical capillary space. The capture zones of the round diagnostic element **6** can be placed in discrete zones or over the entire length of the diagnostic element **6**. The capture zones may also circle the diameter of the diagnostic element **6** or may be applied to only a radius of the diagnostic element **6**. The reaction mixture may be delivered to the diagnostic element **6** through the tube **8**. Furthermore, the cylindrical volume of the tube **8** may be used as a reaction chamber **4** and a disc shaped sample reaction barrier **3** with grooves on its perimeter may also be inserted to form the reaction chamber **4** and the sample addition reservoir **2**. From this discussion, now referring to FIGS. **1** and **2**, one skilled in the art can also appreciate that the flat diagnostic element **6** may also be curved such that the curvature is a radius of a circle.

One skilled in the art can appreciate that various means can be used for the detection of signal at the capture zone of the diagnostic element. In the case of the use of biosensors, such as, for example, a piezoelectric crystal, the piezoelectric crystal onto which would be immobilized a receptor, would be the capture zone and the response generated by binding target ligand would be generally reflected by an electrical signal. Other types of detection means include, but are not limited to visual and instrumental means, such as spectrophotometric and reflectance methods. The inventive features of the diagnostic element described herein allows for improved capture efficiencies on surfaces over which a reaction mixture flows and that various means for detection may be used by one skilled in the art.

The surfaces of the capillaries in the device are generally hydrophilic to allow flow of the sample and reaction mixture through the device. In a preferred embodiment the surface opposing the diagnostic element **6** is hydrophobic such that the reaction mixture repels this surface. The repulsion of reaction mixture to the surface opposing the diagnostic element **6** forces the reaction mixture, and particularly the protein conjugates, to the surface where capture occurs, thus improving the capture efficiency of the components of the reaction mixture to the capture zone. The hydrophobic surfaces opposing the diagnostic element can have a tendency to become hydrophilic as the reaction mixture progresses through the diagnostic element because various components which may be present endogenously or exogenously in the sample or reaction mixture, such as, for example, proteins or polymers, bind to the hydrophobic surface. A preferred hydrophobic surface opposing the diagnostic element can be composed of teflon. It is well known to those skilled in the art that teflon surfaces bind proteins poorly. Thus, the teflon surface opposing the diagnostic element would not become as hydrophilic as would surfaces composed of, for example, polystyrene, polyacrylate, polycarbonate and the like, when the reaction mixture flows through the diagnostic element.

In another preferred embodiment, the diagnostic element **6** is hydrophilic but the areas adjacent to the diagnostic element **6** are hydrophobic, such that the reagents of the assay are directed through only the hydrophilic regions of the diagnostic element. One skilled in the art will recognize that various techniques may be used to define a hydrophilic diagnostic element or zone, such as plasma treatment of hydrophobic surfaces using masks which shield the surfaces, except for the diagnostic element, from the treatment or by application of hydrophobic adhesives to hydrophilic surfaces to define a diagnostic element or by the use of viscous hydrophobic compounds, such as an oil or a grease. In another preferred embodiment, the capillary of the diagnostic element can be formed by ultrasonic welding. The boundaries of the diagnostic element are dictated by the energy directors which are used to form the sonicated weld.

The surfaces of the diagnostic element **6** or of the other components of the device may be smooth or grooved or grooved and smooth. Various textured surfaces may also be employed, alone or in combination with smooth or grooved surfaces. For example, surfaces composed of posts, grooves, pyramids and the like, referred to as protrusions, or holes, slots, waffled patterns and the like, referred to as depressions may be utilized. The textured geometries may be ordered in rows, staggered or totally random and different geometries may be combined to yield the desired surface characteristics. The depressions or the protrusions of the textured geometries can range from about 1 nm to 0.5 mm and preferably from about 10 nm to 0.3 mm. The distance between the various depressions and protrusions can range from about 1 nm to 0.5 mm and preferably from about 2 nm to 0.3 mm.

In a preferred mode as shown in FIGS. **1** and **2**, one surface of the diagnostic element **6** is grooved and the grooves are perpendicular to the flow of the reaction mixture and the opposing surface is smooth. In another embodiment, one surface of the diagnostic element **6** is grooved at the capture zone and the areas adjacent to the capture zone are

smooth. The opposing surface of the diagnostic element **6** may be smooth or may be grooved, for example, the grooves of each surface intermesh. The positioning of the grooves of the diagnostic element perpendicular to the flow of the reaction mixture is beneficial in that the flow of the reaction mixture through the diagnostic element **6** occurs in an organized manner with a distinct, straight front dictated by the grooves in the capillary space. In addition, when one surface is in close proximity, for example 1 μm to 100 μm, to the peaks of the grooves then the capture efficiency of the components from the reaction mixture can be enhanced. The enhancement of capture efficiency at the capture zones in grooved diagnostic elements as compared to smooth surface elements may be related to the movement of the reaction mixture in the capillary space; that is, in the case of the grooved surface the reaction mixture is forced to move over the peak of the groove and into the trough of the next groove. Thus, a finer grooved surface, that, is, more grooves per cm, would provide a better capture efficiency than a coarser grooved surface. The reaction mixture is thus driven closer to the surface of the grooved diagnostic element than it would be if both surfaces were smooth. Also, the close proximity of the surfaces decreases the volume of the bulk reaction mixture above the grooved surface of the diagnostic element and therefore decreases the diffusion distance of the components which bind to the diagnostic element. The proximity of the surfaces of the diagnostic element should minimize the volume of reaction mixture in the diagnostic element at the capture zone without blocking the capillary flow through the element. The capture of, for example, the complex of target ligand: Ligand receptor conjugate at the capture zone can approach 100% efficiency if the proximity of the surfaces is optimized. The capture of nearly all of the ligand receptor conjugate which is bound by target ligand is most desired because a greater sensitivity of the assay as a function of sample volume can be achieved. Other advantages of improved capture efficiency are that less reagents are used because the sample volume is decreased, the assay device can be miniaturized because of the smaller sample volume and the reproducibility of the assay result will be improved because changes in the rate of flow of the reaction mixture through the capture zones will have less or no effect on the capture of the labelled conjugates.

The capillary space can be defined by a variety of ways, for example, machining the surfaces to the appropriate tolerances or using shims between the surfaces. In a preferred embodiment, ultrasonic welding of the surfaces defines the capillary. In this case, the capillary space is defined by the energy directors and the distance between the surfaces is a function of the size of the energy director, the welding energy, the time of energy application and the pressure applied during welding. The surfaces of the diagnostic element can be parallel or non-parallel. In the latter case, the flow rate of the reagents through the diagnostic element will not be uniform throughout the length. A preferred embodiment is to maintain the surfaces of the diagnostic element approximately parallel. The surfaces of the diagnostic element can be made from materials, such as plastics which are capable of being milled or injection molded, for example, polystyrene, polycarbonate, polyacrylate and the like or from surfaces of copper, silver and gold films upon which are adsorbed various long chain alkanethiols as described in J.Am.Chem.Soc. 1992, 114, 1990–1995 and the references therein. In this latter example, the thiol groups which are oriented outward can be used to covalently immobilize proteins, receptors or various molecules or biomolecules which have attached maleimide or alkyl halide groups and which are used to bind components from the reaction mixture for determining the presence or amount of the target ligand.

Referring to FIGS. **3***a* and **3***b*, the zones of immobilization of one or more receptors or the placement of biosensors at the capture zone **17** on the diagnostic element **6** can take many forms. For example, if the target ligand is very low in concentration in the sample then one would desire that all of the reaction mixture pass over the zone of immobilized receptor or biosensor to obtain the best signal from the given volume of reaction mixture. In this case, the placement of the reagents or biosensors on the diagnostic element **6** at the capture zones **17** could, for example, resemble that shown in FIG. **3***a*. If the target ligand in the sample is high in concentration and the sensitivity of the analytical method is not an issue then the placement of the receptors or biosensors at the capture zones **17** could, for example, resemble that in FIG. **3***b*. One skilled in the art can appreciate that the placement of receptors or biosensors on the diagnostic element is a function of the sensitivity requirements of the analytical method.

One or more diagnostic elements can comprise a device. The reaction mixture may be applied to a device with multiple diagnostic elements. In addition, the sample may be applied to the device and then separated into different reaction chambers, each with separate diagnostic elements. The capture zone can be various geometrical symbols or letters to denote a code when the sample is positive or negative for the target ligand. One skilled in the art will recognize the useful combinations of the elements of this invention.

The diagnostic element can also be configured to perform a semi-quantitative or quantitative assay, as for example, is described in Clinical Chemistry (1993) 39, 619–624, herein referred to by reference only. This format utilizes a competitive binding of antigen and antigen label along a solid phase membrane. The improvement is that the use of the diagnostic element described herein for the above cited method would require a smaller sample volume and improved binding efficiency to the solid phase surface.

Diagnostic Elements other than Capillaries

The inventive teachings described herein of the adsorbtion of proteins, particularly receptors to plastic surfaces, can be utilized for adsorbtion of receptors to many plastic surfaces which are not a part of a capillary. Nanoparticles and latex particles coated with receptors can also be applied to surfaces of many types of immuno-assay devices, as for example, to "dipsticks." Dipsticks are generally used as a solid phase onto which are bound, as a result of the assay process, for example, the ligand receptor conjugate. Dipsticks generally incorporate membranes; however, a disadvantage in the use of membranes in dipsticks is the difficulty in washing the unbound ligand receptor from the membrane. Thus, an improvement in the use of dipsticks is to immobilize receptor coated latex or nanoparticles directly onto a plastic surface of the dipstick. The removal of unbound ligand conjugate from the plastic surface is thus more efficient than removal from a membrane.

Used Reagent Reservoir

Referring to FIGS. **1** and **2**, the used reagent reservoir **7** receives the reaction mixture, other reagents and excess sample from the diagnostic element **6**. The volume of the used reagent reservoir **7** is at least the volume of the sample and extra reagents which are added to or are in the device. The used reagent reservoir **7** can take many forms using an absorbent, such as a bibulous material of nitrocellulose, porous polyethylene or polypropylene and the like or the

used reagent reservoir can be comprised of a series of capillary grooves. In the case of grooves in the used reagent reservoir **7**, the capillary grooves can be designed to have different capillary pressures to pull the reagents through the device or to allow the reagents to be received without a capillary pull and prevent the reagents from flowing backwards through the device. The size and quantity of the grooved capillaries determine the volume and capillarity of the used reagent reservoir **7**. In a preferred embodiment, as shown in FIG. **4**, the fingers **52** at the end of the diagnostic element **6** are in fluid contact with a capillary space **55** and the capillary space **55** is in fluid contact with a grooved or textured capillary space **56**. The depth of the grooves or textured surface can be, for example, about 0.1 mm to 0.6 mm, preferably about 0.3 mm to 0.5 mm and the density can range from about 5 to 75 grooves per cm and preferably about 10 to 50 grooves per cm. Referring to FIG. **4**, the reagents of the device move to the fingers **52** at the end of the diagnostic element **51** and into the capillary channel **55**. The reagents either partially or completely fill the capillary space **55** and then come in contact with the grooved or textured surface **56**. The width of the capillary space **55** is generally about 1 mm to 3 mm and the depth is generally about 0.1 mm to 2 mm. The length of the capillary space **55** should be sufficient to be in fluid contact with the grooved or textured surface **56**. The grooved or textured surface **56** partially or completely pulls the reagents from the capillary channel **55** depending on the rate of delivery of the reagents into the capillary space **55** from the diagnostic element **51**. When the flow of reagents is complete in the device, the grooved or textured surface **56** has greater capillarity than the capillary channel **55** and the reagents are removed from the capillary channel **55** by the grooved or textured surface **56**. In addition, the reverse flow of the reagents from the grooved or textured surface is not preferred because the capillarity in the grooved or textured surface **56** holds the reagents and prevents their backward flow. One skilled in the art can recognize from these inventive features that the arrangement of grooves or a used reagent reservoir within the device can be adapted to a variety of desired objectives.

The Description of the One-Step Assay Device

The elements of the device which have been described individually can be assembled in various ways to achieve the desired function. The term "one-step" implies that one manual action is required to achieve the assay result, for example, adding sample to the device is one step. In the case of the device performing a one-step assay which involves both a timed incubation of reagents and a wash step, the wash solution is excess sample and the assay device is built with the elements in fluid communication using the sample addition reservoir, the sample-reaction barrier, the reaction chamber, the time gate, the diagnostic element and the used reagent reservoir as depicted in FIG. **1**. The devices are generally about 3 cm to 10 cm in length, 1 cm to 4 cm in width and about 2 mm to 15 mm thick. Typically, a top member with smooth surfaces is placed onto a bottom member which has a surface onto which are built the elements stated above. The relationship of the elements are as depicted in FIG. **1**. The reagents required for performing the assay are immobilized or placed in the respective elements. The surfaces are brought together, a capillary distance apart, and in doing so, the regions of the sample addition reservoir, the sample reaction barrier, the reaction chamber, the time gate, the diagnostic element, the gap and the used reagent reservoir are all formed and are capable of functioning together. Also, the surfaces are brought together such that the opposing surfaces touch to form and seal the sample addition reservoir, the reaction chamber, and the used reagent reservoir.

When performing a qualitative, non-competitive assay on one or more target ligands, the signal producing reagents, which could include, for example, a receptor specific for the target ligand adsorbed to a colloidal metal, such as a gold or selenium sol, are placed on the sample reaction barrier or in the reaction chamber in dried or lyophilized form. Another receptor for each target ligand is immobilized onto the surface of the diagnostic element at the capture zone. The time gate is positioned generally on the diagnostic element between the reaction chamber and the capture zones by the placement of, for example, a surfactant-free polystyrene suspension onto the device in an amount which dictates the desired incubation time. The incubation time is usually the amount of time for the reactions to come to substantial equilibrium binding. The assay is then performed by addition of sample to the sample addition reservoir of the device. The sample moves over the sample-reaction barrier, into the reaction chamber by the aid of the fingers and dissolves the reagents in the reaction chamber to form the reaction mixture. The reaction mixture incubates for the amount of time dictated by the time gate. The excess sample remaining in the sample addition reservoir and reaction mixture in the reaction chamber are in fluid communication but are not in substantial chemical communication because of the sample-reaction barrier. Thus, the reaction chamber defines the volume of the reaction mixture. The reaction mixture then moves past the time gate and onto the diagnostic element and over the capture zones. The complex of receptor conjugate and target ligand formed in the reaction mixture binds to the respective receptor at the capture zone as the reaction mixture flows over the capture zones. The reaction mixture may also flow over a positive control zone, which can be for example, an immobilized receptor to the signal development element. As the reaction mixture flows through the diagnostic element and into the used reagent reservoir by the aid of the fingers, the excess sample flows behind the reaction mixture and generally does not substantially mix with the reaction mixture. The excess sample moves onto the diagnostic element and removes the receptor conjugate which did not bind to the capture zone. When sufficient excess sample washes the diagnostic element, the signal at the capture zones can be interpreted visually or instrumentally. Referring to FIG. **1***d*, in a preferred mode of the above description, the reaction mixture moves onto the diagnostic element **6**, over the capture zone or zones and then the reaction mixture proceeds into a capillary gap **18**. The capillary gap **18** generally has less capillarity than that of the diagnostic element **6**. The capillary space **19** of the diagnostic element **6** is generally smaller than the capillary space of the gap **18**. The volume of the capillary gap **18** generally approximates the volume of the reaction mixture such that the capillary gap **18** fills slowly with the reaction mixture and once filled, the capillarity of the remaining portion of the diagnostic element **6** or used reagent reservoir is greater than the capillarity of the gap **18** resulting in an increased rate of flow to wash the diagnostic element **6**. As one skilled in the art can appreciate, the gap **18** can be formed in the top member **8** or in the bottom member **9** or a combination of both members **8** and **9**.

In the case of the device performing a one-step assay which does not involve a timed incubation step but does involve a wash step in which the wash solution is excess sample, the assay device is built with the elements in fluid communication using the sample addition reservoir, the sample-reaction barrier, the reaction chamber, the diagnostic

element and the used reagent reservoir. The assay reagents are used as described above for the non-competitive qualitative assay. The assay device without the time gate allows the reaction mixture to flow onto the diagnostic element without an extended incubation time. The capillary flow of the reaction mixture and the excess sample are as described above.

The optional reagent chamber is incorporated into the device in the case of the device performing a one-step assay with the introduction of an additional assay reagent into or after the reaction mixture or the introduction of a wash solution which flows behind the reaction mixture through the device. The optional reagent chamber may be in fluid contact with any element of the device and is generally in fluid contact with the reaction chamber. When in fluid contact with, for example, the reaction chamber, the optional reagent chamber and the reaction chamber may be separated by a time gate. Various reagents may be dried or lyophilized in the optional reagent chamber, such as detergents for a washing step or reagents which are sequentially provided to the diagnostic element after the reaction mixture.

In the case of performing one-step, non-competitive, quantitative assays the reagents as described above for the non-competitive, qualitative assay may apply. The device is comprised of the elements, sample addition reservoir, sample-addition barrier, reaction chamber, time gate, diagnostic element and used reagent reservoir. In this case, the capture zone of the diagnostic element is generally the entire diagnostic element. That is, the capture zone is a length of the diagnostic element onto which the receptor conjugate binds. The receptor conjugate binds along the length of the capture zone in proportion to the amount of target ligand in the sample. The device of the present invention is preferred for this quantitative assay because of the high efficiency of capture of the reagents, for example, the binding of a complex of target ligand and receptor conjugate to an immobilized receptor to the target ligand on the capture zone, and because the movement of the reaction mixture over the diagnostic element proceeds with a sharp front. The receptors on the capture zone sequentially become saturated with the complex of target ligand and receptor conjugate as the reaction mixture moves over the length of the capture zone. The length of the diagnostic element containing bound conjugate then determines the concentration of the target ligand. Those skilled in the art will recognize the format of this type of immunoassay as a quantitative immunochromatographic assay as discussed in U.S. Pat. Nos. 4,883,688 and 4,945,205, hereby incorporated by reference.

In the case of the device performing a one-step, qualitative, competitive assay which involves both a timed incubation of reagents and a wash step and the wash solution is excess sample, the assay device is built with the elements in fluid communication using the sample addition reservoir, the sample-reaction barrier, the reaction chamber, the time gate, the diagnostic element and the used reagent reservoir. When performing a qualitative competitive assay on one or more target ligands, the conjugate is composed of, for example, a ligand analogue coupled to signal development element, such as a gold or selenium sol. The conjugate and receptor for each target, ligand are placed in the reaction chamber in dried or lyophilized form, for example, in amounts which are taught by U.S. Pat. Nos. 5,028,535 and 5,089,391, hereby incorporated by reference. Another receptor for each target ligand is immobilized onto the surface of the diagnostic element at the capture zone. The time gate is positioned generally on the diagnostic element between the reaction chamber and the capture zones as described previously. The incubation time is usually the amount of time for the reactions to come to substantial equilibrium binding. The assay is then performed by addition of sample to the device. The sample moves over the sample-reaction barrier and into the reaction chamber, dissolves the reagents to form the reaction mixture and incubates for the time dictated by the time gate. The excess sample and reaction mixture are in fluid communication but not in substantial chemical communication because of the sample-reaction barrier. The reaction mixture then moves onto the diagnostic element and over the capture zones. The ligand analogue conjugate binds to the respective receptor or receptors at the capture zone or zones. As the reaction mixture flows over the diagnostic element and into the used reagent reservoir, the excess sample flows behind the reaction mixture and generally does not substantially mix with the reaction mixture. The excess sample moves onto the diagnostic element and removes conjugates which do not bind to the capture zone or zones. When sufficient excess sample washes the diagnostic element the results at the capture zones can be interpreted visually or instrumentally. In a preferred mode of the above invention, the reaction mixture moves onto the diagnostic element, over the capture zone or zones and then the reaction mixture proceeds into a capillary gap. The capillary gap has less capillarity than that of the diagnostic element. The volume of the capillary gap generally approximates the volume of the reaction mixture such that the capillary gap fills slowly with the reaction mixture and once filled, the capillarity of the remaining portion of the diagnostic element or used reagent reservoir is greater resulting in an increased rate of flow of excess sample to wash the diagnostic element.

In another aspect of the one-step, competitive assay, the reaction mixture is composed of ligand analogue-ligand complement conjugate to each target ligand and receptors adsorbed to latex particles with diameters of, for example, 0.1 μm to 5 μm to each target ligand, in appropriate amounts, for example, as taught by U.S. Pat. Nos. 5,028,535 and 5,089,391. The ligand complement on the conjugate can be any chemical or biochemical which does not bind to the receptors for the target ligands. The assay is begun by addition of sample to the device. Sample fills the reaction chamber and is incubated for a time which allows the reagents to come to substantial equilibrium binding. The reaction mixture flows over the time gate and onto or into a filter element to prevent ligand analogue-ligand complement conjugates which have bound to their respective receptor latexes from passing onto the diagnostic element. Typical filter elements can be composed of nitrocellulose, cellulose, nylon, and porous polypropylene and polyethylene and the like. Thus, only the ligand analogue-ligand complements conjugate which were not bound by the receptor latex will pass onto the diagnostic element. The receptor to the ligand complement of the conjugate is immobilized on the diagnostic element at the capture zone and binds the conjugate. A wash step may not be required because the filter removes the conjugate bound to latex; however, the excess sample or a wash solution from the optional reagent chamber may be used to wash the diagnostic element.

In the case of a one-step quantitative, competitive assay, the receptor to the ligand analogue conjugate or the ligand complement of the conjugate is immobilized onto the diagnostic element as described previously for the one-step quantitative, non-competitive assay. Thus, the concentration of the target ligand in the sample is visualized by the distance of migration on the diagnostic element of the conjugate. In another mode, a quantitative assay could be performed by the binding of the labelled conjugate, for

example, the ligand analogue-ligand complement conjugate, to sequential, discrete capture zones of receptor on the diagnostic element. The quantitative result is achieved by the depletion of the conjugate as the reaction mixture flows through the capture zones of the diagnostic element.

The Device as a Diagnostic Element

The diagnostic element of the device can be utilized with a sample addition means to perform a separation step of bound and unbound conjugates. An example of this type of device which has a sample addition means, a diagnostic element and a used reagent reservoir is depicted in FIG. **2**. For example, in the case of a non-competitive assay, at least one receptor conjugate is incubated with sample which is suspected of containing at least one target ligand in a suitable vessel and this reaction mixture is applied to the sample addition zone of the device. The reaction mixture then flows onto the diagnostic element and over the capture zone of, for example, immobilized receptor to the target ligand. When target ligand is present in the sample, the target ligand-receptor conjugate complex binds to the receptor on the capture zone. If the signal development element is an enzyme, then either a substrate for the enzyme which produces a visual color or a wash solution followed by a substrate is next added to the device. Excess reagents flow to the used reagent reservoir. The presence or amount of each target ligand in the sample is then determined either visually or instrumentally.

In the case of a competitive immunoassay, for example as taught by U.S. Pat. Nos. 5,028,535 and 5,089,391, herein incorporated by reference, the diagnostic element may be used to separate bound and unbound ligand analogue conjugates such that the unbound ligand analogue conjugates bind to the receptors of the diagnostic element in proportion to the presence or amount of target ligand in the sample.

One skilled in the art can appreciate that all formats of immunoassays or gene probe assays which require a separation step of free and bound conjugates or the separation of free of bound reagents which subsequently leads to the ability to detect a signal can utilize the inventive features of the diagnostic element. One skilled in the art can also recognize that the inventive elements of this invention, namely, the fingers, the sample reaction barrier, the reaction chamber, the time gate, the diagnostic element, the fluid control means and the used reagent reservoir can be used separately or in various combinations and in conjunction with other devices not described here. For example, the sample reaction barrier with fingers and the reaction chamber can be used in conjunction with devices incorporating porous members, such as membranes to deliver precise volumes of reagents to the porous member. The time gate can also be incorporated into the aforementioned devices or the time gate may be used alone in conjunction with devices incorporating porous members. The fluid control means can also be used in devices incorporating porous members to control the rate of flow of reagents through the porous member.

EXPERIMENTAL PROCEDURES

Example 1

Preparation of Anti-βhCG Antibody-Colloidal Gold Conjugate

Colloidal gold with an average diameter of 45 nm was prepared according to the method of Frens, Nature, Physical Sciences, 241, 20 (1973). The colloidal gold conjugate was prepared by first adding 5.6 ml of 0.1 M potassium phosphate, pH 7.58, dropwise with rapid stirring to 50 ml of colloidal gold. Anti β-subunit monoclonal antibody to hCG (Applied Biotech, San Diego, Calif.; 1 ml of 4.79 mg/ml in phosphate buffered saline, 0.02% sodium azide, pH 7) was added in a bolus to the colloidal gold with rapid stirring. After complete mixing the stirring was stopped and the solution was incubated at room temperature for 1 h. Polyethylene glycol (average molecular weight=20,000) was added (0.58 ml) as a 1% solution to the colloidal gold solution and the solution was mixed. The colloidal gold solution was subjected to centrifugation at 27,000 g and 5° C. for 20 min. The supernatant was removed and each pellet was washed twice by resuspension and centrifugation with 35 ml of 10 mM potassium phosphate, 2 mM potassium borate, 0.01% polyethylene glycol (average molecular weight=20,000), pH 7. After the final centrifugation, the pellet was resuspended in 0.5 ml of the wash buffer. The gold conjugate was diluted for the assay of hCG into a buffered solution containing 10 mg/ml bovine serum albumin at pH 8.

Example 2

Preparation of Anti-αhCG Antibody Latex

Surfactant-free polystyrene particles (Interfacial Dynamics Corp., Portland, Oreg.; 0.106 ml of 9.4% solids, 0.4 μm) was added while vortexing to anti α-subunit hCG monoclonal antibody (Applied Biotech, San Diego, Calif.; 0.89 ml of 6.3 mg/ml in 0.1 M 2-(N-morpholino) ethane sulfonic acid, (MES), pH 5.5) and the suspension was incubated at room temperature for 15 min. The suspension was subjected to centrifugation to pellet the latex particles. The pellet was washed three times by centrifugation and resuspension of the pellet with 10 mM MES, 0.1 mg/ml trehalose, pH 5.5. The final pellet was resuspended in the wash buffer at a solids concentration of 1%.

Example 3

Preparation of Goat Anti-Mouse Latex

Surfactant-free polystyrene particles (Interfacial Dynamics Corp., Portland, Oreg.; 0.11 ml of 9.4% solids, 0.6 μm) were added while vortexing to goat IgG antibody against mouse IgG (Jackson ImmunoResearch Laboratories, Inc.; 0.89 ml of 0.34 mg/ml in 0.1 M MES, pH 5) and the suspension was incubated at 45° C. for 2 h. The suspension was subjected to centrifugation to pellet the latex particles. The pellet was washed three times by centrifugation and resuspension of the pellet with 10 mM MES, 0.2 mg/ml trehalose, pH 5.5. The final pellet was resuspended in the wash buffer at a solids concentration of 1%.

Example 4

Prearation of the One-Step Device for a Qualitative hCG Assay

A one-step device made of plastic was built having an 80 to 100 μl sample addition reservoir, a 20 μl reaction chamber and a 40 μl used reagent reservoir. This device is designed for applying samples of about 20 μl to 100 μl, but the reaction chamber is fixed at 20 μl. In cases where a larger reaction mixture volume is required for the desired assay, then the reaction chamber would be increased to that volume and the sample addition reservoir would be about 2 to 4 times the volume of the reaction chamber volume. The devices were plasma treated to graft functional groups which create a hydrophilic surface. Those skilled in the art will recognize that the plasma treatment of plastic is performed in a controlled atmosphere of a specific gas in a high frequency field. The gas ionizes, generating free radicals which react with the surface. The sample addition reservoir was shaped as a trapezoid with dimensions of 14 mm and 7 mm for the parallel sides and 7 mm for the other sides with

a depth of 0.49 mm. The sample addition reservoir was adjacent to the sample reaction barrier. The sample-reaction barrier was 1.5 mm long and 7 mm wide including grooves running parallel to the flow of the sample at a density of 50 grooves per cm and a depth of 0.1 mm. In the case of sample volumes larger than 20 to 80 μl, the width of the reaction barrier and thereby the reaction chamber could be increased to accommodate the desired flow rate but the groove size or density could remain as indicated. The fingers in the walls of the reaction chamber and the used reagent reservoir were 1 m wide and 0.4 mm deep with 7 fingers in each wall of the reaction chamber and the used reagent reservoir. The reaction chamber volume was 20 μl. The reaction chamber was shaped as a trapezoid with dimensions of 7 mm and 3.5 mm for the parallel sides and 7.1 mm for the other sides with depths of 0.56 mm for 20 μl reaction chambers. The diagnostic element was about 2.5 cm long, 2 mm wide and 1 mm from the base of the device including grooves running perpendicular to the flow of reaction mixture at a density of 100 grooves per cm and a depth of 0.05 mm. In the case of a time gate on the diagnostic element, the time gate was positioned on the diagnostic element immediately adjacent to the reaction chamber. The width of the diagnostic element could be increased to increase the flow of the reaction mixture to the desired rate past the capture zones. The anti-αhCG antibody latex (1 μl) and the goat anti-mouse latex (1 μl) were applied to the diagnostic element of the devices approximately 1.5 cm apart. The anti-βhCG antibody colloidal gold conjugate (10 μl) was pipetted into the trough of the reaction chamber. The devices were placed under vacuum for about 15 min. to dry the reagents. The used reagent reservoir had the shape of a trapezoid with dimensions of 7 mm and 15 mm for the parallel sides and 8 mm for the other sides with a depth of 0.5 mm. Referring to FIG. **4**, in a preferred (best mode) embodiment of the used reagent reservoir, the reaction mixture moved to a capillary space **55** (1.25 mm long, 27.5 mm wide and 0.48 mm deep) from the diagnostic element **6**, aided by fingers **52** (1 mm wide and 0.4 mm deep with 7 fingers), and then into a grooved capillary structure (13.6 mm long, 25.4 mm wide, 0.61 mm deep with a density of 16 grooves per cm). The outer walls and the top surface of the walls of the sample addition reservoir and the reaction chamber had applied a thin coating of silicon grease to prevent the leakage of the reagents from the reservoir and chamber of the assembled device. The capillary spaces in the devices were then formed by placing a clear plastic polycarbonate sheet on top of the device. The plastic sheet was held to the opposing surface with binder clips. The clear plastic sheet had a sample port above the sample addition reservoir for the introduction of sample.

Example 5

Qualitative One-Step Assay for hCG

The devices described in Example 4 were used for the qualitative one-step assay for hCG. The assay times for the devices without the time gates were about 5 to 10 min. A urine solution (60 μl) containing 0, 50, 200 and 500 mIU hCG/ml was added to the sample reservoir of the devices. The sample moved into the reaction chamber, dissolved the colloidal gold conjugate and the reaction mixture moved onto the diagnostic element over the anti-hCG latex and goat anti-mouse IgG latex capture zones. The reaction mixture moved into the used reagent reservoir and the excess sample washed the diagnostic element. The color density of the capture zones for hCG was measured instrumentally using a Minolta Chroma Meter CR 241 at 540 nm. A red color was visible for samples containing hCG and not visible for the sample without hCG at the capture zones for hCG. The ΔE* values for the 0, 50, 200 and 500 mIU/Ml were 0, 7.78, 12.95 and 20.96, respectively, and for the positive control (goat anti-mouse IgG) zones a distinctive red bar was observed with a ΔE* of about 35.

Example 6

Qualitative One-Step Assay for hCG Using a Time Gate

Devices as described in Example 4 were prepared with the addition of the time gate. The time gate was formed on the diagnostic element which is in contact with the reaction mixture in the reaction chamber. The time gate was prepared by adding 1 μl of 2% solids of surfactant-free, sulfated latex, 1.0 μm, (Interfacial Dynamics Corp., Portland, Oreg. The other reagent latexes and gold conjugate were also added to the devices and dried as described in Example 5. Clear plastic sheets were placed on the devices and sample (about 60 μl) containing 0, 50, 200 and 500 mIU hCG/ml was added to the devices. The sample moved into the reaction chamber, dissolved the colloidal gold conjugate and the reaction mixture remained in the reaction chamber for about 8 to 10 min, whereas in devices without time gates the reaction mixture remained in the reaction chamber for 5 sec to 15 sec. The proteinaceous components of the reaction mixture, which may be present in the sample and which was added as a component of the reaction mixture, namely, bovine serum albumin, bound to the latex particles of the time gate and changed the hydrophobic surface of the time gate into a hydrophilic surface. Other proteins, such as gelatin, serum albumins, immunoglobulins, enzymes and the like and polypeptides and hydrophilic polymers will also function to bind to the hydrophobic zone. The gradual transformation of the hydrophobic surface to a hydrophilic surface, which resulted through binding of the proteinaceous components of the reaction mixture to the latex particles allowed the reaction mixture to flow over the area of the time gate. In control experiments in which protein, namely bovine serum albumin, was not added to the reaction mixture, flow of the reaction mixture over the time gate and onto the diagnostic element did not occur during the time (5 h) of the experiment. This control experiment showed that the urine sample alone did not contain sufficient protein or components which bind to the applied latex of the time gate to allow a change in the hydrophobic character of the time gate. In the event that the components in the sample should only be used to cause the transformation of the hydrophobic time gate to a hydrophilic one for the reaction mixture to flow, then one would be required th lower the mass and total surface area of the latex applied to the time gate to an extent which would allow flow of the reaction mixture over the time gate in an appropriate amount of time. The reaction mixture then moved onto the diagnostic element over the anti-hCG latex and goat anti-mouse IgG latex capture zones. The reaction mixture moved into the used reagent reservoir and the excess sample washed the diagnostic element. The color density of the capture zones for hCG was measured instrumentally using a Minolta Chroma Meter CR 241. A red color was visible for samples containing hCG and not visible for the sample without hCG at the capture zones for hCG. The ΔE* values for the 0, 50, 200 and 500 mIU/ml were 0, 6.51, 13.14 and 18.19, respectively. A red color bar was visible at the goat anti-mouse IgG capture zones of each device.

Example 7

Qualitative One-Step Assay for hCG Using a Flow Control Means

Devices as described in Example 4 were prepared with the addition of the optional flow control means. The optional

flow control means or "gap" was placed behind the capture zone for hCG gold conjugate on the diagnostic element. The gap between the two surfaces was 0.38 mm, the length of the gap was 13.2 mm and the width of the gap on the top member was 9 mm; however, the effective width of the gap was the width of the diagnostic element (2 mm). This gap volume above the diagnostic element was about 10 μl which was, in this case, half the volume of the reaction chamber. The anti-hCG and the goat anti-mouse latexes and gold conjugate were added to the device and dried as described in Example 5. Clear plastic sheets of polycarbonate having a gap in one surface were placed on the devices with the gap facing the diagnostic element.

Sample (about 60 μl) containing 0 and 200 mIU hCG/ml was added to the devices. The sample moved into the reaction chamber, dissolved the colloidal gold conjugate and-the reaction mixture then moved onto the diagnostic element over the anti-hCG latex. The reaction mixture then entered the gap which was immediately behind the capture zone of anti-hCG latex. The flow rate over the capture zone slowed while the reaction mixture moved over the capture zone and filled the gap. The time for the 10 μl reaction mixture to fill the gap was about 12 min to 16 min, whereas with devices without the optional flow control means, the times were about 1 min to 3 min. for the reaction mixture to pass over the capture zone. When the reaction mixture filled the gap, the reaction mixture then moved into the narrow capillary of the diagnostic element and over the goat anti-mouse capture zone. The reaction mixture moved into the used reagent reservoir and the excess sample washed the diagnostic element. The color density of the capture zones for hCG was measured instrumentally using a Minolta Chroma Meter CR 241. A red color was visible for samples containing hCG and not visible for the sample without hCG at the capture zones for hCG. The ΔE* values for the 0 and 200 mIU/ml were 0 and 16.12. The δE* value of the hCG capture zone for the device without the flow control means for the 200 mIU/ml sample was 16.32. A red color bar was visible at the goat anti-mouse IgG capture zones of each device.

Example 8

Preparation of the Diagnostic Element for Multi-step Assays

A device was built comprising a sample addition reservoir and a diagnostic element. The devices were plasma treated to graft functional groups which create a hydrophilic surface. The sample addition reservoir had dimensions of 12 mm long, 6 mm wide and 0.05 mm deep. The diagnostic element was about 5.5 cm long, 1.3 mm wide and 1 mm from the base of the device and included grooves running perpendicular to the flow of reaction mixture at a density of 100 grooves per cm and a depth of 0.05 mm. In the case of qualitative assays, the antibody latex (1 μl) was applied to the diagnostic element, covering the entire width and 1 cm length of the diagnostic element. In the case of an immunochromatographic assay, the antibody latex (6 μl) was applied to the entire width and length of the diagnostic element. The devices were placed under vacuum for about 1 h to dry the reagents. The capillary spaces in the device were then formed by placing a clear plastic polystyrene sheet on top of the device. The plastic sheet was held to the opposing surface with binder clips.

Example 9

Assay for hCG Using the Diagnostic Element

The diagnostic element described in Example 8 was used for the assay of hCG. Urine samples (20 μl) containing 0, 50, 200 and 500 mIU/ml hCG were added to tubes containing anti-βhCG antibody colloidal gold conjugate (2 μl). The tubes were vortexed and the reaction mixtures were incubated for 5 min at room temperature. The reaction mixtures (20 μl) were applied in 10 μl aliquots to the sample addition reservoir of the device. The reaction mixture flowed onto the diagnostic element from the sample reservoir and over the capture zone. An absorbent at the end of the capture zone removed the used reagent from the diagnostic element. The color density of the capture zones for hCG was measured instrumentally using a Minolta Chroma Meter CR 241. A red color was visible for samples containing hCG and not visible for the sample without hCG at the capture zones for hCG. The ΔE* values for the 0, 50, 250 and 500 mIU/ml were 0.00, 1.24, 3.16 and 5.56, respectively.

Example 10

Synthesis of meta-Nitrophencyclidine

To an ice cooled solution of, phencyclidine hydrochloride (5 g, 1.8×10^{-2}mol) in concentrated sulfuric acid (9 ml) was added dropwise, and with stirring, fuming nitric acid (2 ml). The reaction mixture was stirred in an ice-water bath for 1 hour and then poured onto crushed ice/ water. The mixture was made basic with 10N sodium hydroxide (50 ml) to pH12 and extracted with diethyl ether (2×100 ml). The combined organic layers were washed with water (2×100 ml), dried over anhydrous magnesium sulfate, filtered and evaporated under vacuum. The residue was treated with methyl alcohol (20 ml) and heated on a hot water bath (80° C.) until solute dissolved. The flask was covered with aluminum foil (product is light sensitive) and the solution was allowed to stir at room temperature overnight when a yellow solid precipitated. The solid was collected by filtration and dried under vacuum to afford 3.0 g (58%) of m-nitrophencyclidine as fine yellow crystals which were protected from light: mp 81–82° C.

Example 11

Synthesis of meta-Aminophencyclidine

To a stirring solution of m-nitrophencyclidine (3.0 g, 10.4×10^{-3}mol) in methyl alcohol (150 ml) was added, under a flow of argon, 10% palladium-carbon (0.5 g) followed by ammonium formate (4.0 g, 6.3×10–2mol). The reaction mixture was stirred at room temperature for 2 hours after which time the catalyst was removed by filtration and the solvent was evaporated under vacuum. The residue was treated with iN potassium hydroxide solution (30 ml) and extracted with diethyl ether (2×50 ml). The combined organic extracts were washed with water (50 ml), dried over anhydrous magnesium sulfate, filtered and evaporated under vacuum. The residue was dissolved in hexane (20 ml) and the solution was stirred at room temperature overnight when a white solid precipitated. The solid was collected by filtration and dried under vacuum to afford 1.4 g (52%) of m-aminophencyclidine: mp 121–122° C.

Example 12

Synthesis of Acetylthionropionic Acid

To a stirred solution of 3-mercaptoproprionic acid (7 ml, 0.08 moles) and imidazole (5.4 g, 0.08 moles) in tetrahydrofuran (THF, 700 ml) was added dropwise over 15 minutes, under argon, a solution of 1-acetyl imidazole (9.6 g, 0.087 moles) in THF (100 ml). The solution was allowed to stir a further 3 hours at room temperature after which time the THF was removed in vacuo. The residue was treated with ice-cold water (18 ml) and the resulting solution acidified with ice-cold concentrated HCl (14.5 ml) to pH 1.5–2. The mixture was extracted with water (2×50 ml), dried over magnesium sulfate and evaporated. The residual

crude yellow oily solid product (10.5 g) was recrystallized from chloroform-hexane to afford 4.8 g (41% yield) acetylthiopropionic acid as a white solid with a melting point of 44–45° C.

Example 13

Synthesis of meta-Acetylthioprolionamide Phencyclidine

To a stirring solution of m-aminophencyclidine (1.4 g, 5.4×10^{-3}mol) and acetylthiopropionic acid (0.87 g, 5.8×10^{-3}mol) in anhydrous tetrahydrofuran (7 ml) was added dicyclohexylcarbodiimide (1.19 g, 5.8×10^{-3}mol). The flask was purged with argon and the solution stirred at room temperature for 2 hours. The mixture was filtered from insoluble dicyclohexylurea and evaporated under vacuum. The residual solid was recrystallized from chloroform/hexane to afford 1.5 g (71%) of m-acetylthiopropionamide phencyclidine as a white crystalline solid: mp 152–4° C.

Example 14

Synthesis of meta-3-Mercaptoroprionamide Phencyclidine

meta-Acetylthiopropionamide phencyclidine (0.01 g, 2.57×10^{-5} mol) was dissolved in 1.29 ml 0.12M potassium carbonate in 80% methanol/20% water (v/v). The solution sat at room temperature for 5 min andbhen 0.2 ml 0.5 M potassium phosphate, pH 7, was immediately added and the solution was adjusted to pH 7–7.5 with hydrochloric acid (1 N). The title compound in solution was used as is to react with BSA-SMCC.

Example 15

Preparation of Phencyclidine Analogue Attached to Bovine Serum Albumin (BSA-PCP)

Bovine serum albumin (BSA, 3.5 ml of 20 mg/ml) was reacted with succinimidyl 4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (SMCC, Pierce Chemical Co.) by adding a solution of 6.7 mg SMCC in 0.3 ml acetonitrile and stirring the solution at room temperature for 1 h while maintaining the pH between 7 and 7.5 with 1N potassium hydroxide. The protein was separated from unreacted compounds by gel filtration chromatography in 0.1 M potassium phosphate, 0.02 M potassium borate, 0.15 M sodium chloride, pH 7.0. The meta-3-mercaptoproprionamide phencyclidine (0.2 ml of 13 mM) was added to the BSA-maleimide (2 ml at 8.2 mg/ml) and the solution was stirred at room temperature for 4 h. The solution was then dialyzed 3 times against 1000 ml of 10 mM MES, pH 5.5. Recover 1.8 ml BSA-PCP at 8 mg/ml.

Example 16

Preparation of Phencyclidine Analogue Colloidal Gold Conjugate

A solution (4.7 ml) containing BSA (22 mg) and BSA-PCP (5.6 mg) in 10 mM MES, pH 5.5 was added in a bolus to colloidal gold (105 ml) in 10 mM MES, pH 5.5 with rapid stirring. After complete mixing the stirring was stopped and the solution was incubated at room temperature for 1 h. The colloidal gold conjugate was subjected to diafiltration against 50 mM potassium phosphate, 10 mM potassium borate, pH 7, using a tangential flow device (Sartorius Easy Flow, molecular weight cutoff was 100,000) to remove BSA and BSA-PCP which was not bound to colloidal gold. The gold conjugate was diluted for the assay of PCP into a buffered solution containing 10 mg/ml bovine serum albumin at pH 7.5.

Example 17

Preparation of Anti-Phencyclidine Antibody Latex

Surfactant-free polystyrene particles (Interfacial Dynamics Corp., Portland, Oreg.; 0.074 ml of 9.4% solids, 0.4 μm) was added while vortexing to anti-phencyclidine monoclonal antibody (0.926 ml of 5.86 mg/ml in 0.1 M MES, pH 5) and the suspension was incubated at 45° C. for 2 h. The suspension was subjected to centrifugation to pellet the latex particles. The pellet was washed three times by centrifugation and resuspension of the pellet with 10 mM MES, 0.1 mg/ml trehalose, pH 5.5. The final pellet was resuspended in the wash buffer at a solids concentration of 1%.

Example 18

Preparation of Latex-Immobilized Affinity-Purified Goat IgG Antibody Against the Fc Fragment of Mouse IgG (Goat Anti-mouse Fc Latex)

Affinity-purified goat anti-mouse (Fc (Immunosearch) and polystyrene latex particles (sulfated, 1.07 μm) (Interfacial Dynamics) were incubated separately at 45° C. for one hour, the antibody solution being buffered with 0.1 M 2-(N-morpholino) ethane sulfonic acid at pH 5.5. While vortexing the antibody solution, the suspension of latex particles was added to the antibody solution such that the final concentration of antibody was 0.3 mg/ml and the solution contained 1% latex solids. The suspension was incubated for 2 hours at 45° C. prior to centrifugation of the suspension to pellet the latex particles. The latex pellet was resuspended in 1% bovine serum albumin in phosphate-buffered-saline (PBS) and incubated for one hour at room temperature. Following centrifugation to pellet the latex, the pellet was washed three times by resuspension in PBS and centrifugation. The final pellet was resuspended in PBS containing 0.1% sodium azide at pH 7.0 at a latex concentration of 1% solids.

Example 19

Assay for Phencyclidine Using the Diagnostic Element

The diagnostic element described in Example 8 was used for the assay of phencyclidine (PCP). Urine samples (133 μl) containing 0, 100, 200 and 300 ng/ml PCP were added to tubes containing a lyophilized buffer formulation (containing 10 mM potassium phosphate, 150 mM sodium chloride and 10 mg/ml BSA, pH 8) and phencyclidine analogue colloidal gold conjugate (4 μl) was added and the solution was vortexed. Anti-PCP antibody (2.8 μl of 0.1 mg/ml) was added to each tube and the solutions were vortexed and incubated at room temperature for 5 min. Goat anti-mouse Fc latex (50 ml of a 1% suspension) was added to the tubes, the tubes were vortexed and incubated at room temperature for 10 min. The solutions were then filtered to remove the complex of the PCP analogue gold conjugate: anti-PCP antibody:goat anti-mouse latex from the reaction mixture using a Gelman Acrodisc® 3 syringe filter (0.45 μm). The filtrates of the reaction mixtures (20 μl) were applied to the diagnostic elements described in example 8. The reaction mixture flowed onto the diagnostic element from the sample reservoir and over the capture zone. An absorbent tissue placed 1 cm after the capture zone removed the used reagent from the diagnostic element. The color density of the capture zones was measured-itrumentally using a Minolta Chroma Meter CR 241. The ΔE^* values for the 0, 100, 200 and 300 ng/ml samples were 0.69, 9.28, 14.04 and 21.6, respectively.

Although the foregoing invention has been described in some detail by way of illustration and example, it will be obvious that certain changes or modifications may be practiced within the scope of the appended claims. As used herein, references to "preferred" embodiments refer to best modes for practicing the invention.

I claim:

1. An assay device comprising:

i. a sample addition reservoir positioned so that one side of said sample addition reservoir is adjacent to a sample reaction barrier (ii);

ii. a sample reaction barrier between said sample addition reservoir and said reaction chamber, said barrier having a capillarity greater than the capillarity of said reaction chamber, whereby absent an externally applied force, fluid flows from the sample reaction barrier to the reaction chamber pursuant to capillary force;

iii. a reaction chamber adapted for receiving fluid from said sample reaction barrier, said chamber comprising a wall which comprises at least two fingers;

iv. a time gate positioned immediately adjacent to the reaction chamber to receive fluid into said diagnostic element;

v. a diagnostic element capable of immobilizing at least one conjugate in at least one zone; and.

vi. a used reagent reservoir disposed a capillary space away from said diagnostic element, so that fluid flow is directed into said capillary space.

2. Device of claim 1 wherein said diagnostic element further comprises fingers positioned downstream from said zone or zones to prevent fluid from flowing backwards over said zone(s).

3. Device of claim 1 wherein said diagnostic element is comprised of a non-porous surface.

4. An assay device comprising:

i. a sample addition reservoir;

ii. a reaction chamber;

iii. a sample reaction barrier between said sample addition reservoir and said reaction chamber, said sample reaction barrier having a greater capillarity than said reaction chamber, wherein said reaction chamber is adapted to receive fluid flow from said sample reaction barrier;

iv. a wall perpendicular or substantially perpendicular to fluid flow in said sample reaction barrier, said wall located at the interface between said sample reaction barrier and said reaction chamber, said wall comprising grooves perpendicular or substantially perpendicular to fluid flow in said sample reaction barrier, said grooves having widths of between 0.5 mm to 2 mm wide and 0.1 mm to 1.5 mm in depth, whereby absent an externally applied force, fluid flows from the sample reaction barrier to the reaction chamber pursuant to capillary force;

iv. a time gate for delaying fluid flow from said reaction chamber to a separation element (v), said time gate located between said reaction chamber and said separation element, said time gate comprising at least one hydrophobic surface which is capable of binding at least one component present in said fluid; and whereby the delay of fluid flow is related to the rate of binding of the component to said hydrophobic surface whereby binding of the component to said hydrophobic surface changes said hydrophobic surface into a hydrophilic surface whereby fluid can flow into said separation element;

v. a separation element capable of entrapping, or removing by binding at least one component from said fluid;

vi. a time gate for delaying fluid flow from said separation element to a diagnostic element (vii) so as to maximize desired binding interactions in said diagnostic element, said time gate located between said separation element and said diagnostic element, said time gate comprising at least one hydrophobic surface which is capable of binding at least one component present in said fluid; and whereby the delay of fluid flow is related to the rate of binding the component to said hydrophobic surface whereby binding of the component to said hydrophobic surface changes said hydrophobic surface into a hydrophilic surface whereby fluid can flow into said diagnostic element (vii);

vii. a diagnostic element capable of immobilizing at least one conjugate in at least one zone; and,

viii. a used reagent reservoir.

5. Device of claim 4 wherein said means (vi) is a nonporous or porous surface.

6. Device of claim 4 wherein said diagnostic element (viii) is a nonporous or porous surface.

7. Diagnostic assay device comprising:

i. a sample addition reservoir;

ii. a sample reaction barrier between said sample addition reservoir and a reaction chamber (iii), said sample reaction barrier comprising a first capillary;

iii. the reaction chamber, said reaction chamber comprising a second capillary, said second capillary adapted to receive fluid flow from said first capillary, said first capillary having a greater capillarity than said second capillary;

iv. a wall perpendicular or substantially perpendicular to fluid flow in said first capillary, said wall located at the interface between said first capillary and said second capillary, said wall comprising grooves substantially perpendicular to fluid flow in said first capillary, said grooves having widths of between 0.5 mm to 2 mm wide and 0.1 mm to 1.5 mm in depth, whereby absent an externally applied force, fluid flows from the sample reaction barrier to a reaction chamber pursuant to capillary force;

v. a membrane comprising a time gate, said time gate for delaying fluid flow from said membrane to a diagnostic element (vi), said time gate immobilized in said membrane, with said time gate located next to, or a capillary distance from, said diagnostic element, said time gate comprising at least one hydrophobic surface which is capable of binding at least one component present in said fluid; and whereby the delay of fluid flow is related to the rate of binding the component to said hydrophobic surface whereby binding of the component to said hydrophobic surface changes said hydrophobic surface into a hydrophilic surface whereby fluid can flow into said diagnostic element;

vi. a diagnostic element capable of immobilizing at least one conjugate in at least one zone; and,

vii. a used reagent reservoir.

8. An assay device comprising:

i. a sample addition reservoir;

ii. a reaction chamber;

iii. a sample reaction barrier between said sample addition reservoir and said reaction chamber, said sample reaction barrier having a greater capillarity than said reaction chamber, wherein said reaction chamber is adapted to receive fluid flow from said sample reaction barrier;

iv. a wall perpendicular or substantially perpendicular to a fluid flow direction in said sample reaction barrier, said wall located at the interface between said sample reaction barrier and said reaction chamber, said wall comprising grooves perpendicular or substantially per-

pendicular to the fluid flow direction in said sample reaction barrier, said grooves having widths of between 0.5 mm to 2 mm wide and 0.1 mm to 1.5 mm in depth, whereby absent an externally applied force, fluid flows from the sample reaction barrier to a reaction chamber pursuant to capillary force; and.

v. a diagnostic element capable of immobilizing at least one conjugate in at least one zone, said diagnostic element fluidly connected to said reaction chamber.

9. The device of claim **8** further comprising a used reagent reservoir, fluidly connected to said diagnostic element.

10. The device of claim **8** wherein the sample addition reservoir comprises a filter element capable of removing particulate matter from a sample.

11. The device of claim **8** further comprising a means for entrapping, or removing by binding, at least one component from a fluid.

12. The device of claim **8** wherein said diagnostic element is a nonporous surface or a porous surface.

13. The device of claim **8** further comprising a time gate that can delay flow of a fluid.

14. The device of claim **13** wherein the time gate is located on a hydrophilic surface between said reaction chamber and said diagnostic element, or on a hydrophobic surface between said reaction chamber and said diagnostic element.

15. The device of claim **13** wherein the time gate is adapted to allow fluid flow from said reaction chamber to said diagnostic element through said time gate by capillary action, surface tension, hydrostatic pressure or a combination thereof.

16. The device of claim **13** wherein the time gate is positioned between said reaction chamber and said diagnostic element, wherein the time gate delays fluid flow from said reaction chamber to the diagnostic element, said time gate comprising at least one hydrophobic surface which is capable of binding at least one component present in said fluid; and whereby the delay of fluid flow is related to the rate of binding the component to said hydrophobic surface, whereby binding of the component to the hydrophobic surface changes said hydrophobic surface into a hydrophilic surface whereby fluid can advance.

17. An assay device for detecting at least one target ligand in a fluid sample, the device comprising:

a first capillary region and a second capillary region, said first capillary region having a greater capillarity than said second capillary region; and,

a wall perpendicular or substantially perpendicular to a fluid flow direction in said first capillary region, said wall located at an interface between said first capillary region and said second capillary region, said wall comprising grooves perpendicular or substantially perpendicular to the fluid flow direction in said first capillary region, wherein said grooves are between 0.5 mm to 2 mm wide and 0.1 mm to 1.5 mm deep, whereby absent an externally applied force, fluid flows from the first capillary region to the second capillary region pursuant to capillary force.

18. An assay device for detecting at least one target ligand in a fluid sample, said assay device comprising:

a. a sample addition reservoir;

b. a time gate for delaying fluid flow;

c. a diagnostic element; wherein said assay device is configured and arranged such that said fluid sample flows from said sample addition reservoir to said time gate to said diagnostic element.

19. The assay device of claim **18**, wherein said time gate comprises at least one hydrophobic surface.

20. The assay device of claim **18**, wherein said time gate comprises hydrophobic poly-electrolytes.

21. The assay device of claim **18**, wherein said time gate is V-shaped.

22. The assay device of claim **18**, wherein said diagnostic element is clear.

23. The assay device of claim **18**, wherein said diagnostic element immobilizes said target ligand for detection.

24. The assay device of claim **18**, comprising a used reagent reservoir configured and arranged after said diagnostic element.

25. The assay device of claim **18**, wherein said diagnostic element comprises latex particles.

26. The assay device of claim **25**, wherein said latex particles have a diameter between 0.01 μm and 10 μm.

27. The assay device of claim **18**, wherein said time gate comprises latex particles.

28. The assay device of claim **27**, wherein said latex particles have a diameter between 0.01 μm and 10 μm.

29. The assay device of claim **27**, wherein said protrusion is 1 nm to 0.5 mm.

30. The assay device of claim **18**, wherein said time gate comprises at least one groove.

31. The assay device of claim **30**, wherein said groove is parallel to the direction of flow of said fluid sample.

32. The assay device of claim **30**, wherein said groove is perpendicular to the direction of flow of said fluid sample.

33. The assay device of claim **18**, comprising a reaction chamber configured and arranged between said time gate and said diagnostic element.

34. The assay device of claim **33**, wherein said reaction chamber is 0.05 mm to 10 mm deep.

35. The assay device of claim **33**, wherein said reaction chamber comprises a ramp.

36. The assay device of claim **35**, wherein said ramp is at an angle between 25 degrees and 45 degrees relative to the floor of said reaction chamber.

37. The assay device of claim **18**, comprising a sample-reaction barrier configured and arranged between said sample reservoir and said time gate.

38. The assay device of claim **37**, wherein said sample-reaction barrier comprises more than one groove between 10 and 500 grooves per centimeter.

39. The assay device of claim **37**, wherein said sample-reaction barrier comprises at least one groove.

40. The assay device of claim **39**, wherein said groove is perpendicular to the direction of flow of said fluid sample.

41. The assay device of claim **39**, wherein said groove is parallel to the direction of flow of said fluid sample.

42. The assay device of claim **39**, wherein said groove is 0.01 mm to 0.5 mm deep.

43. The assay device of claim **39**, wherein said groove is 0.5 mm to 2 mm wide.

44. The assay device of claim **18**, wherein said diagnostic element comprises at least one groove.

45. The assay device of claim **44**, wherein said groove is parallel to the direction of flow of said fluid sample.

46. The assay device of claim **44**, wherein said groove is perpendicular to the direction of flow of said fluid sample.

47. The assay device of claim **44**, wherein said groove is a protrusion.

48. The assay device of claim **44**, wherein said groove is spaced between 1 nm and 0.5 mm apart from another groove.

49. The assay device of claim **44**, wherein said groove is a depression.

50. The assay device of claim **49**, wherein said depression is 1 nm to 0.5 mm.

* * * * *

Appendix C: Related Proceedings Appendix

None.